Complex Regulation of Pax6 Neuronal Progenitors
By Rb Family Members During Corticogenesis
Benedetta Naglieri

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
in the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY
2012
ABSTRACT

Complex Regulation of Pax6 Neuronal Progenitors By Rb Family Members During Corticogenesis

Benedetta Naglieri

The retinoblastoma tumor suppressor (pRB) inhibits tumorigenesis by restraining cell cycle progression via repression of the E2F transcription factor family and by promoting cell differentiation via activation of lineage-specific transcription factors. In contrast, the closely related pRB homologues, p107 and p130, are known to inhibit cell cycle progression by repressing the E2F transcription factor family, but are not known to have roles in promoting cell differentiation. Interestingly, the Rb promoter contains a critical cassette of binding sites (Sp1/Ets, ATF and E2F) that is conserved between mice and humans. Previously, our lab developed a wild type Rb promoter–LacZ transgenic reporter line (T157) that displayed dynamic and neuronal-specific expression (Agromayor et al., 2006). We generated mutant Rb promoter-LacZ transgenic lines and demonstrated that the conserved cassette controls Rb expression, positively through the Sp1/Ets site and negatively through the E2F site. Repression of the Rb promoter through this critical E2F site means that the E2F family lies both upstream and downstream of Rb, and suggests that Rb family members regulate the Rb promoter during neuronal development.

To identify which Rb family member represses the Rb promoter during corticogenesis, we generated Rbp-LacZ lines in genetic backgrounds deficient in various Rb family members and looked for deregulation of Rbp-LacZ activity within the embryo (Aim 1). Surprisingly, Rbp-LacZ activity responds in opposing ways with either loss of Rb or dual loss of p107 and p130, demonstrating that regulation of the Rb promoter by Rb family members during corticogenesis is complex.

To determine whether direct or indirect mechanisms are responsible for the opposing changes in Rbp-LacZ expression with loss of Rb family members in the developing cortex, we evaluated occupancy at the Rb promoter (ChIP analysis), proliferation, cell death (BrdU incorporation and TUNEL analysis) and changes in gene expression (RT-PCR) in wild type vs. mutant cortices from embryos lacking various Rb family members (Aim 2). Interestingly, we found evidence for both direct and indirect action of Rb family member inactivation on the Rb promoter.

To determine if the opposing changes in Rbp-LacZ activity with either loss of Rb or dual loss of p107 and p130 occurs in a cell autonomous or a non-cell autonomous manner, we optimized and
analyzed primary cortical neuron cultures from wild type and mutant embryos to quantitate $RbP\text{-}LacZ$ activity on a cell-by-cell basis (Aim 3). We compared changes in the frequency and intensity of $RbP\text{-}LacZ$ activity, the distribution of neuronal subpopulations, identified the cells expressing $RbP\text{-}LacZ$ activity and evaluated differences in these populations with loss of various $Rb$ family members. Through these studies, we have discovered a complex relationship exists between $Rb$ family members and Pax6 progenitors during corticogenesis, underscoring the intricate nature of the network connecting the $Rb$ and $E2f$ families in vivo.
# TABLE OF CONTENTS

**List of Figures** ........................................................................................................................................vi

**Acknowledgements** .................................................................................................................................x

## Chapter 1: Introduction ...............................................................................................................................1

Identification of *RB* as the First Tumor Suppressor ..................................................................................1

The *RB* Tumor Suppressor Pathway ...........................................................................................................4

The *RB* Family of “Pocket Proteins” .........................................................................................................4

*RB* Family Regulation of E2F Transcription ..............................................................................................5

*RB* Family Members have Distinct Functions In Cell Cycle Regulation ....................................................7

*RB* Mediates Transcriptional Repression Through Recruitment of Chromatin Modifiers .......................8

*RB* Promotes Differentiation Through Interaction with Tissue Specific Factors .....................................9

Mouse Models Reveal Specific Functions of *RB* Family Members .............................................................10

Mouse Models of Retinoblastoma ................................................................................................................13

*Rb* Family Expression During Development and Differentiation ................................................................14

Regulation of *Rb* Expression .....................................................................................................................15

Specific Aims ...............................................................................................................................................20

## Chapter 2: *RbP-LacZ* Activity in Embryos Lacking Various *Rb* Family Members ...............................22

In Situ Analysis of *RbP-LacZ* Activity in Embryos at E14.5 ..................................................................22

Analysis of *RbP-LacZ* reporter in either *p107- and p130-deficient E14.5 embryos* ................................22

Analysis of *RbP-LacZ* reporter with dual loss of *p107 and p130* in E14.5 embryos .................................24

Analysis of *RbP-LacZ* reporter in *Rb* conditional knockout E14.5 embryos ............................................27

Analysis of *RbP-LacZ* reporter in *Meox2Cre;RbLoxP/LoxP* embryos on a mixed genetic background ..........................27

Analysis of *RbP-LacZ* reporter in *Meox2Cre;RbLoxP/LoxP* embryos on an inbred C57BL/6 background ..........................................................27

Excision efficiency driven by *Meox2-Cre vs. Nestin-Cre in the CNS* ....................................................32

Analysis of *RbP-LacZ* reporter with neuronal specific loss of *Rb* in E14.5 embryos ............................35

In Situ Analysis of *RbP-LacZ* Activity in the Isolated Cortex .................................................................35

*RbP-LacZ* activity in the wild type *T157* cortex at E14.5 and in the adult ................................................35
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>RbP-LacZ activity in the <em>p130</em>-deficient cortex</td>
<td>38</td>
</tr>
<tr>
<td>RbP-LacZ activity with combined loss of <em>p107</em> and <em>p130</em> in the E14.5 cortex</td>
<td>41</td>
</tr>
<tr>
<td>RbP-LacZ activity in the conditionally <em>Rb</em>-deficient cortex at E14.5</td>
<td>41</td>
</tr>
<tr>
<td>Summary</td>
<td>44</td>
</tr>
<tr>
<td>Chapter 3: Direct Versus Indirect Effects of <em>Rb</em> Family Inactivation on the <em>Rb</em> Promoter</td>
<td>46</td>
</tr>
<tr>
<td>Direct Effects of <em>Rb</em> Family Members on the <em>Rb</em> Promoter</td>
<td>46</td>
</tr>
<tr>
<td>ChIP analysis of the <em>Rb</em> promoter within wild type embryonic and juvenile cortex</td>
<td>48</td>
</tr>
<tr>
<td>ChIP analysis of the <em>Rb</em> promoter in the E14.5 embryonic cortex</td>
<td>48</td>
</tr>
<tr>
<td>ChIP analysis of the <em>Rb</em> promoter in the juvenile cortex</td>
<td>50</td>
</tr>
<tr>
<td>Analysis of the <em>Rb</em> promoter by ChIP in various <em>Rb</em> family deficient cortices at E14.5</td>
<td>52</td>
</tr>
<tr>
<td>Analysis of the <em>Rb</em> promoter in <em>Rb</em>-deficient cortex</td>
<td>52</td>
</tr>
<tr>
<td>Analysis of the <em>Rb</em> promoter in <em>p107</em>-deficient cortex</td>
<td>54</td>
</tr>
<tr>
<td>Analysis of the <em>Rb</em> promoter in <em>p130</em> and *p107;<em>p130</em>-deficient cortex</td>
<td>54</td>
</tr>
<tr>
<td>Evaluating Indirect Effects of <em>Rb</em> Family Member Loss on <em>Rb</em> Promoter Activity</td>
<td>56</td>
</tr>
<tr>
<td>Changes in cortical size with loss of <em>Rb</em> family members</td>
<td>56</td>
</tr>
<tr>
<td>Changes in proliferation in various <em>Rb</em> family deficient cortices at E15</td>
<td>58</td>
</tr>
<tr>
<td>Effect of <em>p107</em> or <em>p130</em> loss on proliferation in the E15 cortex</td>
<td>58</td>
</tr>
<tr>
<td>Effect of dual loss of <em>p107</em> and <em>p130</em> on proliferation in the E15 cortex</td>
<td>61</td>
</tr>
<tr>
<td>Effect of conditional loss of <em>Rb</em> on proliferation in the E15 cortex</td>
<td>65</td>
</tr>
<tr>
<td>Changes in apoptosis in various <em>Rb</em> family deficient cortices at E15</td>
<td>65</td>
</tr>
<tr>
<td>Effect of loss of <em>p107</em> or <em>p130</em> on cell death in the E15 cortex</td>
<td>65</td>
</tr>
<tr>
<td>Effect of dual loss of <em>p107</em> and <em>p130</em> on cell death in the E15 cortex</td>
<td>68</td>
</tr>
<tr>
<td>Effect of conditional loss of <em>Rb</em> on cell death in the E15 cortex</td>
<td>68</td>
</tr>
<tr>
<td>Investigation of changes in gene expression with various <em>Rb</em> family member loss</td>
<td>68</td>
</tr>
<tr>
<td>Effects of <em>Rb</em> family member loss on regulators of neuronal development</td>
<td>71</td>
</tr>
<tr>
<td><em>Rb</em> family member expression in cortex lacking various <em>Rb</em> family members</td>
<td>71</td>
</tr>
<tr>
<td><em>E2f</em> family member expression in cortex lacking various <em>Rb</em> family members</td>
<td>74</td>
</tr>
<tr>
<td><em>p53</em> family member expression in cortex lacking various <em>Rb</em> family members</td>
<td>76</td>
</tr>
</tbody>
</table>
Chapter 4: RbP-LacZ Activity in Primary Cortical Culture with Loss of Various Rb Family Members

Development of a Primary Cortical Culture from the T157 RbP-LacZ E14.5 Cortex

Generation of a 2-dimensional neuronal specific culture

Characterization of proliferation in E14.5 primary cortical cultures

Identification of Diverse Populations in Primary Cortical Neuron Cultures

Progenitor subpopulations in primary cortical neuron cultures

Proliferation in neuronal progenitor populations

Mature neuronal populations in primary cortical cultures

Analysis of RbP-LacZ Activity in E14.5 Primary Cortical Neuron Cultures

RbP-LacZ activity in wild type T157 cortical cultures

RbP-LacZ activity in primary cortical culture lacking various Rb family members

Effect of Rb Family Member Loss on Proliferation in Primary Cortical Cultures

Comparison of Neuronal Populations in Cortical Cultures Lacking Various Rb Family Members

Neurogenesis still occurs with loss of various Rb family members

Presence of mature neuronal subtypes in primary cultures lacking various Rb family members

Presence of neuronal progenitors in Rb family member deficient cultures

Unexpected regulation of the Pax6 progenitor populations by Rb family members

Identification of the RbP-LacZ Expressing Subpopulations in Primary Cortical Cultures

Detection of neuronal maturity and LacZ activity through co-staining

Detection of neuronal progenitors and LacZ activity through co-staining

Changes in RbP-LacZ Activity in Neuronal Subpopulations with Loss of Rb Family Members

RbP-LacZ activity in neuronal subpopulations with loss of p107 or p130

RbP-LacZ activity in neuronal subpopulations with dual loss of p107 and p130

Effect of dual loss of p107 and p130 on the frequency of RbP-LacZ expressing Pax6+ cells

Effect of dual loss of p107 and p130 on the intensity of RbP-LacZ in Pax6+ cells

RbP-LacZ activity in neuronal subpopulations with loss of Rb
Effect of Rb loss on RbP-LacZ activity in progenitor populations.................................122

Changes in nuclear size with Rb loss in primary cortical cultures.................................122

Summary......................................................................................................................................126

Chapter 5: Discussion..................................................................................................................130

Rb Family Members Regulate the Rb Promoter In Vivo..........................................................130

   pRB acts directly and indirectly to repress Rb expression in the developing cortex...........130

   p107 and p130 activate Rb expression in a cell-autonomous manner..................................131

Rb Family Members and Pax6 Share a Close Relationship In Neuronal Development........133

Rb Family Members Vary in Function During Neuronal Development..................................137

Increase in Nuclear Size and Polyploidy with Rb-Deficiency..............................................139

Future directions.........................................................................................................................140

Summary......................................................................................................................................143

Chapter 6: Materials and Methods..........................................................................................146

Generation of Mice......................................................................................................................146

Genotyping by PCR analysis........................................................................................................147

Embryo Collection......................................................................................................................150

Tissue Collection.........................................................................................................................150

Xgal Staining of Whole Mount Embryos..................................................................................150

Xgal Staining of Whole Mount Brains........................................................................................150

BrdU Incorporation......................................................................................................................151

TUNEL..........................................................................................................................................151

Quantitative RT-PCR Analysis...................................................................................................152

Chromatin Preparation from Embryonic Cortex .....................................................................152

Chromatin Preparation from Adult Cortex.............................................................................154

Chromatin Immunoprecipitation...............................................................................................155

Amplification of ChIP DNA.........................................................................................................157

Primary Cortical Neuron Culture..............................................................................................157

Xgal Staining of Cortical Cultures............................................................................................158
EdU Proliferation Assay ................................................................................................................. 159
Immunofluorescence .......................................................................................................................... 159
Co-Xgal/Immunofluorescence ............................................................................................................ 160
Determination of Saturation in Co-Xgal/Immunofluorescence Stained Cultures ......................... 162
Measurement of Nuclear Area in Primary Neuron Culture ............................................................... 162
References ........................................................................................................................................ 163

Appendix:
Quantification of Rb Family Member Binding at the Rb Promoter ............................................... 194
Increase in the intensity of Pax6 staining in Rb deficient cortical culture on Day 4 ......................... 196
Figures and Tables
Figure 1-1: The RB tumor suppressor is a key regulator of the cell cycle and differentiation.................2
Figure 1-2: Structure of mammalian RB family members .................................................................6
Figure 1-3: Rb1 promoter sequence is highly conserved between mouse and human.........................16
Figure 1-4: Structure of the RbP-LacZ transgene........................................................................18
Figure 1-5: Patterns of LacZ staining in Rb promoter mutant reporter mice....................................19
Figure 2-1: Loss of p107 does not deregulate RbP-LacZ activity at E14.5.......................................23
Figure 2-2: Loss of p130 does not deregulate RbP-LacZ activity at E14.5.......................................23
Figure 2-3: Decreased Rb promoter activity with p107 and p130 deficiency at E14.5.......................25
Table 2-1: Decrease in RbP-LacZ activity with dual loss of p107 and p130 at E14.5 and E15.5 ....26
Figure 2-4: De-repression of the Rb Promoter with conditional loss of Rb at E14.5 on a C57BL/6xFVB background ........................................................................................................28
Table 2-2: Increase in RbP-LacZ activity with conditional loss of Rb using Meox2Cre on a C57BL/6xFVB background. .................................................................29
Figure 2-5: Increased RbP-LacZ activity with conditional Rb loss at E14.5 on a C57BL/6 background .........................................................................................................................30
Table 2-3: Increase in RbP-LacZ activity with conditional loss of Rb using Meox2Cre on a C57BL/6 background ...........................................................................................................31
Figure 2-6: Efficient activation of the ROSALox-STOP-Lox reporter in the CNS by Nestin-Cre at E16.5...............................................................................................................................33
Figure 2-7: Verifying loss of Rb through Cre mediated excision of the RbLoxP locus .....................34
Figure 2-8: Increased RbP-LacZ activity with conditional Rb loss in the CNS at E14.5.................36
Table 2-4: Increase in RbP-LacZ activity with conditional loss of Rb in the CNS using Nestin-Cre......37
Figure 2-9: RbP-LacZ activity within the cortex increases with developmental age.......................39
Figure 2-10: RbP-LacZ activity is seen within different regions of the adult cortex..........................40
Figure 2-11: Moderate decrease in RbP-LacZ activity in p130/- cortex at E14.5............................42
Figure 2-12: Decreased RbP-LacZ activity in the E14.5 cortex with loss of p107 and p130...........43
Figure 2-13: Neuronal specific loss of Rb increases RbP-LacZ activity in the cortex at E14.5...........45
Figure 3-1: Conserved E2F site of the Rb promoter lies within a GC rich region upstream of exon 1

Figure 3-2: Rb family members occupy the Rb promoter in E14.5 wild type cortex

Figure 3-3: E2F and pRB family members occupy the Rb Promoter in the juvenile cortex

Figure 3-4: Moderate changes at the Rb Promoter with loss of Rb in E14.5 cortex

Figure 3-5: Moderate changes at the Rb Promoter with loss of p107 in E14.5 cortex

Figure 3-6: Small changes at Rb promoter in p130/- and p107/-; p130/- cortex at E14.5

Table 3-1: Changes in cortical area with loss of Rb family members at E14.5

Figure 3-7: Development of the laminated structure of the neocortex is a dynamic process

Figure 3-8: Loss of p107 or p130 leads to a moderate decrease in proliferation in the E15 cortex

Figure 3-9: Dual loss of p107 and p130 leads to a moderate increase in proliferation in the E15 cortex

Figure 3-10: Ectopic proliferation with conditional loss of Rb in the E15 cortex

Figure 3-11: Decreased cell death with loss of p107 in the developing cortex at E15

Figure 3-12: Decreased cell death with loss of p130 in developing cortex at E15

Figure 3-13: Decreased cell death with dual loss of p107 and p130 in developing cortex at E15

Figure 3-14: No change in cell death with conditional loss of Rb in the E15 cortex

Table 3-2: Minimal Fgf, Fgfr or Hes family deregulation in E14.5 mutant cortex by RT-PCR

Table 3-3: Absence of deregulation of Rb family members by RT-PCR

Table 3-4: Deregulation of E2f family members in E14.5 cortex

Table 3-5: Minimal deregulation of p53 family members with loss of Rb family members

Figure 4-1: Establishment of E14.5 primary cortical neuron culture

Figure 4-2: A low level of proliferation is maintained in E14.5 cortical neuron culture

Figure 4-3: Various neuronal progenitors are found in E14.5 cortical neuron culture on Day 4

Figure 4-4: Pax6 and Sox2 progenitors are proliferating in cortical neuron culture on Day 4

Figure 4-5: Nestin progenitors are proliferating in cortical neuron culture on Day 4

Figure 4-6: Primary cortical culture consists of predominately neuronal cell types on Day 4
Figure 4-7: A variety of mature neuronal subtypes are present in cortical neuron culture on Day 4.

Figure 4-8: RbP-LacZ activity increases in frequency and intensity with time in culture.

Figure 4-9: No significant changes in RbP-LacZ activity in the various Rb family deficient cortical neuron cultures.

Figure 4-10: Loss of Rb leads to increased proliferation in cortical neuron culture on Day 2.

Figure 4-11: Pax6 progenitors are proliferating in Rb deficient cortical cultures on Day 2.

Figure 4-12: Primary cortical cultures, in the absence of FGF, maintain neuronal identity with loss of various Rb family members.

Figure 4-13: Decrease in reelin+ neurons in p107-/-;p130-/- cortical culture on Day 4.

Figure 4-14: No changes in the frequency of Nestin or Sox2 progenitors in Rb family deficient cortical cultures on Day 4.

Figure 4-15: Decrease in the frequency of Pax6+ cortical progenitors with loss of Rb on Day 4.

Figure 4-16: Rb family members may regulate the Pax6 P1 promoter in the E14.5 cortex.

Figure 4-17: A subset of mature neurons shows RbP-LacZ activity in primary cortical cultures on Day 6.

Figure 4-18: A fraction of interneurons and Cajal-Retzius neurons show RbP-LacZ activity.

Figure 4-19: Pax6 progenitors represent the majority of Xgal+ cells in Day 6 cortical culture.

Figure 4-20: RbP-LacZ expressing cells represent a subset of mature neurons but the majority are Pax6+ neuronal progenitors.

Figure 4-21: The frequency of Xgal+Pax6+ cells decreases with dual loss of p107 and p130.

Figure 4-22: RbP-LacZ intensity is decreased in Xgal+Pax6+ cells with loss of p107 and p130.

Figure 4-23: Loss of Rb leads to an increase in nuclear size in cortical culture on Day 6.

Figure 4-24: An increase in nuclear size is specific to Rb deficiency in Day 4 culture.

Figure 4-25: Loss of Rb can increase RbP-LacZ activity by both cell-autonomous and non-cell autonomous mechanisms.

Figure 4-26: p107 and p130 function as activators of RbP-LacZ expression in a cell autonomous manner.

Figure 5-1: Pax6 and Rb function in similar tissues in both mouse and humans.

Figure 5-2: A model of the complex interactions of Rb family members in Pax6+ progenitors.
Figure 6-1: Determination of genotypes for T157, p107, and p130 embryos

Table 6-1: Primers used for genotyping

Table 6-2: Primers for quantitative RT-PCR

Table 6-3: Antibodies used for ChIP

Table 6-4: Primers and protocols used for ChIP analysis

Table 6-5: Primary and secondary antibodies used for immunofluorescence

Figure A-1: Quantification of ChIP of Rb family members at the Rb promoter

Figure A-2: Increase in the intensity of Pax6 staining in Rb deficient cortical culture on Day 4
Acknowledgements

I would like to thank our technician, Elzbieta Wloga, for her assistance in generating the gene expression data. I would like to thank our undergraduate researcher, Randan Pan, for her assistance in immunohistochemical work. I would also like to thank Sandra Leung for her advice throughout my project. I would like to thank the members of my committee for sacrificing their time and giving me the opportunity to present my work. I would like to thank Dr. Yamasaki for her help and guidance, and believing in my work.
Chapter 1: Introduction

The retinoblastoma gene (*RB1*), the prototypical tumor suppressor, is one of the fundamental regulators of the cell cycle. The function of the retinoblastoma protein (pRB), to negatively regulate cell proliferation, is disrupted in a majority of human tumors. pRB is a key decision maker during cell differentiation, a function which is conserved within the retina of the mouse (D. Chen et al., 2004; MacPherson et al., 2004), the vulva of *C. elegans* (Lu and Horvitz, 1998), and the root meristem of *Arabidopsis* (Wyrzykowska et al., 2006; Borghi et al., 2010), amongst a multitude of other examples, implicating that there is more to the function of RB than tumor suppression. The dual roles of pRB, as regulator of cell cycle progression as well as promoter of differentiation, are thought to be the main reasons loss of pRB function occurs so frequently during tumorigenesis (Figure 1-1).

**Identification of RB as the First Tumor Suppressor**

The discovery of the function of *RB* as a tumor suppressor came about from a combination of genetic analysis of human tumorigenesis and work on DNA tumor viruses. Human retinoblastoma, a pediatric eye tumor, presents clinically as two different forms: an early onset heritable form which is inherited in an autosomal dominant fashion and involves the formation of multiple tumor foci in both eyes (multifocal and bilateral), and a sporadic form that is typically a single tumor found in one eye (unifocal and unilateral). While hereditary retinoblastoma is diagnosed in very young children (mean age = 11 months), sporadic retinoblastoma is most often diagnosed in slightly older children (mean age = 22 months) (Lohmann, 2010). To explain the differences in the kinetics of tumor formation amongst bilateral and unilateral patients, Alfred Knudson proposed the “Two Hit Hypothesis” (Knudson, 1971). Specifically, Knudson hypothesized that children with bilateral retinoblastoma inherited one non-functional allele of a putative *RB* gene from one of their parents, and therefore only a single mutational event was needed for tumorigenesis, explaining the faster onset of bilateral and multifocal nature of retinoblastoma in this group. In contrast, tumorigenesis in sporadic retinoblastoma patients required two distinct mutational events to occur in the same cell, an inherently slower and less probable occurrence. This would also explain why the inherited forms of retinoblastoma present as an autosomal dominant disorder, although, as later discovered, require the loss of both copies of *RB* (autosomal recessive).
Figure 1-1: The RB tumor suppressor is a key regulator of the cell cycle and differentiation. The RB protein acts as modulator of transcription factors important for cell cycle regulation as well as development. pRB acts as a tumor suppressor by inhibiting cell cycle progression, through repression of E2F activity, as well as by promoting differentiation through interactions with tissue specific factors.
The effort to elucidate a genetic basis for the Two Hit Hypothesis led to the identification of the human \textit{RB1} gene (hereafter referred to as \textit{RB}). Recurrent loss of heterozygosity (LOH) at chromosomal locus 13q14 in retinoblastoma samples has been found (Knight et al., 1978; Yunis and Ramsay, 1978; Sparkes et al., 1980). Using this as a guide, positional cloning identified the locus of the putative \textit{RB} gene (Godbout et al., 1983; Cavanee et al., 1983). Cloning of the \textit{RB} gene from hereditary retinoblastoma patients showed that while one allele of the \textit{RB} gene was mutated in both normal blood and tumor cells, the remaining functional allele was lost in retinoblastoma samples, either by fine mutation or gross chromosomal loss, confirming that loss of both copies of \textit{RB} is necessary for the development of retinoblastoma (Friend et al., 1986; W.H.Lee et al., 1987a; Dunn et al., 1988). Additional studies on adult sporadic cancers have shown that \textit{RB} loss is not restricted to retinoblastoma. Inactivating mutations at the \textit{RB} locus have been found in small cell lung cancer (Salgia and Skarin, 1998; Caputi et al., 2005), prostate adenocarcinoma (Bookstein et al., 1990; Brooks et al., 1995; Balk and Knudsen, 2008) and osteosarcoma (Wadayama et al., 1994; Kansara and Thomas, 2007) in adults, demonstrating that \textit{RB} is indeed a tumor suppressor in a variety of tissues.

One mechanism by which \textit{RB} functions as a tumor suppressor was unveiled from studies with DNA tumor viruses. The Adenovirus E1A, SV-40 large T-Antigen, and HPV E7 proteins transform cells by interaction with a number of cellular proteins (Linzer and Levine, 1979; A.E. Smith et al. 1979; Rowe et al., 1983; Harlow et al., 1986) and the pRB protein was identified as one of these interacting proteins. Mutations within the shared LxCxE motif (where L = leucine, C = cysteine, E = glutamic acid, and x = any amino acid) of the viral proteins (Whyte et al., 1988; DeCaprio et al., 1989; Dyson et al., 1989), disrupted interaction with pRB, and inhibited the virus’ ability to transform cells. Ludlow et al. (1990) observed that pRB was hypophosphorylated in G0/G1 of the cell cycle, and it was this hypophosphorylated form that was bound to the SV40 large T-antigen. It was hypothesized that the under-phosphorylation of pRB somehow inhibited progression from G1 to the S-phase of the cell cycle, and that viral oncoproteins, by binding pRB, can overcome this inhibition (reviewed in DeCaprio, 2009). These data tied together the tumor suppressor function of \textit{RB} from human retinoblastoma data with viral-induced transformation, and pointed to pRB as one of the essential proteins in preventing tumorigenesis.
The RB Tumor Suppressor Pathway

One of the primary ways in which RB inhibits tumorigenesis is by inhibiting entry into S-phase. pRB is maintained in a hypophosphorylated form in G0/G1 (Mihara et al., 1989; P.L. Chen et al., 1989; Buchkovich et al., 1989). Mitogenic signals activate the Cyclin-Cdk complexes (Cyclin D with Cdk4 or Cdk6 and Cyclin E with Cdk2), which phosphorylate pRB on crucial serine and threonine residues (Lees et al., 1991; Hinds et al., 1992; Ferreira et al., 1993), and disrupt the associations of pRB with a number of cellular proteins (Lunberg and Weinberg, 1998; Harbour et al. 1999).

INK4 family of CDK inhibitors (INK4A – INK4D) act upstream of Cyclin D/Cdk complexes and prevent Cyclin D binding to and activation of Cdk4/Cdk6, thereby inhibiting the phosphorylation of pRB (Serrano et al., 1993; Guan et al., 1994; Hirai et al., 1995). Additionally, the CIP/KIP family of Cdk inhibitors (\(p27^{kip1}\), \(p57^{kip2}\), and \(p21^{cip1}\)) prevents activation of CyclinE/Cdk2 complexes in G1 and Cyclin A/Cdk2 or Cdk1 complexes in S and G2 (Harper et al., 1993; Polyak et al., 1994; M.H. Lee et al., 1995; Toyoshima and Hunter, 1994; Blain et al., 1997; Aleem et al., 2005). Both mutations in the genes encoding these components, as well as deregulated expression of each of these components, have been found that disrupt function in human tumors, resulting in the identification of key tumor suppressors (e.g., \(INK4A\), \(INK4B\), \(KIP1\)) and oncogenes (e.g., \(CCND1\), \(CCNE1\)) (reviewed in Nevins, 2001; Sherr and McCormick, 2002).

This alternating network of tumor suppressors and oncogenes, referred to as the RB pathway, is deregulated in most human cancers, either by loss of the tumor suppressor function of pRB or the Cdk inhibitors, or by activation of the oncogenic Cyclin/Cdk complexes (reviewed in Sherr and McCormick, 2002). These mutational events are mutually exclusive within tumors, suggesting that additional mutations affecting genes within the RB pathway are not advantageous. For example, while ~80% of small cell lung cancers have mutations in RB, loss of \(INK4A\) occurs in ~60% of non-small cell lung cancer, leading to the idea that loss of a functional RB pathway is one of the critical steps in tumorigenesis.

The RB Family of “Pocket Proteins”

The pRB protein is part of a family of proteins referred to as the "pocket proteins." In mammals, there are three members: pRB, p107, and p130, derived from the \(RB1\), \(RBL1\), and \(RBL2\) genes,
respectively. (To avoid confusion due to the similar nomenclature, \(RB\) family members will be referred to as \(RB\) (\(Rb\)-1), \(p107\) (\(RBL\)-1), and \(p130\) (\(RBL\)-2)). They are grouped based on the presence of a so-called pocket domain, consisting of two large conserved sub-domains designated A and B separated by a variable spacer (Kaelin et al., 1990; H.Y. Kim and Cho, 1997) (Figure 1-2). Phosphorylation of serine and threonine residues by Cyclin-Cdk5 occurs both in the pocket region as well as in the C-terminal domain (Lees et al., 1991; Connell-Crowley et al., 1997; Knudsen and Wang, 1997). The large majority of mutations in retinoblastoma are found within either the A or B sub-domains and rarely within the spacer region (Lohmann, 1999). The domain of \(pRB\) that binds the LxCxE motif, which allows for \(pRB\) to interact with many co-factors as well as the viral oncoproteins, is found within the B sub-domain of the pocket (J.O. Lee et al., 1998; H.Y. Kim et al., 2001). It is this same pocket domain that allows for the \(RB\) family members to bind to E2F transcription factors (S. Huang et al., 1992; C. Lee et al., 2002; Rubin et al., 2005).

Structural analysis of \(pRB\) bound to the LxCxE peptide from the HPV protein showed that E7 and E2Fs can be bound to \(pRB\) in mutually exclusive manners (J.O. Lee et al., 1998), and fine mutation of the residues required for LxCxE binding does not disrupt binding of E2F family members (Dick et al., 2000). The \(p107\) and \(p130\) proteins are more closely related to each other than to \(pRB\) and they both contain a CyclinA/CyclinE-binding domain (between residues 651 and 669) that is absent from \(pRB\) (Ewen et al., 1992; Mayol et al., 1993; reviewed in Classon and Dyson, 2001).

**RB Family Regulation of E2F Transcription**

The most well-characterized proteins that interact with \(pRB\) family members are the E2F transcription factors. E2Fs are a family of transcription factors defined by their DNA binding domain, which recognizes the consensus E2F sequence, \(TTT(G/C)(G/C)CGC\), or non-consensus sequence BKTSSCGS (where \(B = \text{not A}, K = G\) or \(T\), \(S = G\) or \(C\)) (Rabinovitch et al., 2008). They were first discovered by their ability to trans-activate the adenovirus E2 promoter (Kovesdi et al., 1986; Thalmeier et al., 1989; Blake and Azizkhan, 1989; Dynlacht et al., 1994a). There are now eight E2F proteins in mammals that share homologous DNA binding domains. The E2F1-E2F5 proteins all contain a pocket protein binding domain and a dimerization domain for interaction with DP proteins (reviewed in Trimarchi and Lees, 2002). The E2F6-E2F8 proteins do not contain a pocket protein binding domain, and while
Figure 1-2: Structure of mammalian RB family members.

The RB family members are defined by the shared homology within the A and B “pocket domains”, which are necessary for binding the viral oncoproteins, as well as most other interacting proteins. Cell cycle regulated phosphorylation of RB family members occurs at serine/threonine residues primarily, but not exclusively, within the C-terminal domain. The family members p107 and p130 are more closely related to each other than to pRB, with strong sequence homology within the N-terminal and “spacer” regions, including a conserved CyclinA/E Cdk binding site.
E2F6 contains a DP dimerization domain (Trimarchi et al., 1998), E2F7 and E2F8 contain two DNA binding domains, allowing them to function independently of DP protein interaction (DeBruin et al., 2003a; DiStefano et al., 2003; Maiti et al., 2005; Christensen et al., 2005; Logan et al., 2005).

By binding E2F, pRB family members can regulate the activity of E2F target genes. The initial E2F targets studied included factors needed for cell cycle control and DNA synthesis, for example CCNE1 and CDC6 (Ohtani et al., 1995, Botz et al., 1996; Geng et al., 1996; Hateboer et al., 1998; Yan et al., 1998). This agreed with the model that RB family members bind E2F transcription factors in G0/G1, preventing them from activating genes needed for cell cycle progression. The Cyclin/CDK dependent phosphorylation of RB family members releases E2Fs, allowing them to activate the expression of genes required for S-phase progression. Subsequent studies have shown that the types of genes that are E2F targets encompass a broad range of function, including genes involved in the G2/M checkpoint (e.g. MAD2L1 and TTK), DNA damage (e.g. RAD51 and FEN1), apoptosis (e.g. TP73 and CASP3) and chromatin modification (e.g. EZH2 and EED) (Ishida et al., 2001; Weinmann et al., 2002; Ren et al., 2002; Seelan et al., 2002; Irwin et al., 2000; Muller et al., 2001).

**RB Family Members have Distinct Functions In Cell Cycle Regulation**

Although RB family members show high homology within the pocket region needed to bind E2Fs, they each show a preferred subset of E2F binding partners. The E2F proteins interact with RB family members through interactions between the C-terminal domain of pRB with the E2F-DP marked box domains, a highly conserved region shared by E2F family members which mediates various protein interactions, and between the pocket domain and the E2F transactivation domain (Helin et al., 1992; C. Lee et al., 2002; Xiao et al., 2003; Rubin et al., 2005; Burke et al., 2010). E2F1, E2F2, and E2F3a interact exclusively with pRB and are designated as “activator” E2Fs because of their ability to activate transcription of genes required of S-phase entry and cell cycle progression (Takahashi et al., 2000). In contrast, while pRB is found in complexes with E2F4, p107 and p130 are more frequently found in complexes with E2F4 at E2F-regulated promoters (Beijersbergen et al., 1994; Vairo et al., 1995; Moberg et al, 1996; Balciunate et al., 2005). E2F5 is thought to interact exclusively with p130 (Hijmans et al., 1995). The binding of E2Fs for different targets is cell cycle regulated. Inactivation of pRB at the G1/S transition frees “activator” E2Fs to positively regulate their own transcription as well as genes needed to
promote S-phase. The increase in “activator E2Fs” drives cells into S-phase, and as cells enter G2, RB family independent E2Fs (E2F6, E2F7, and E2F8) are thought to mediate the repression of the “activator E2Fs” to prevent ectopic DNA replication (Lees et al., 1993; Leone et al., 1998; L. Wu et al., 2001; Christensen et al., 2005; DiStefano et al., 2003). E2F4 and E2F5 are expressed throughout the cell cycle, but are exported from the nucleus in G1 (Muller et al., 1997; Verona et al., 1997; Gaubatz et al., 2001; Apostolova et al., 2002). Instead of acting as activators of gene expression, E2F4 and E2F5 are thought to repress gene expression, mostly in G0 and early G1, by forming repressor complexes with p107 or p130 (Sardet et al., 1995; Lindeman et al., 1997; Muller et al., 1997; Zini et al., 2001; Balciunatė et al., 2005).

In addition to the different interactions of RB family members with different E2F family members, RB family members are also differentially expressed during the cell cycle. While RB levels remain relatively constant during the cell cycle (Buchkovich K et al., 1989; Ikeda et al., 1996; Burkhart et al., 2010a), levels of p107 and p130 change in a reciprocal manner. While p130 levels remain high in quiescent cells, levels drop rapidly as cells enter G1 (E.J. Smith et al., 1996). On the other hand, p107 levels are relatively low in quiescent cells and entry into G1 leads to an increase in p107 levels (Kiess et al., 1995, Beijerbergen et al., 1995). In fact, p107 is known to regulate a distinct group of E2F responsive genes specifically in cycling cells (Balciunatė et al., 2005). Therefore the different functions of the pRB family of pocket proteins appear to be regulated by both differences in binding partners as well as expression patterns.

RB Mediates Transcriptional Repression Through Recruitment of Chromatin Modifiers

RB family members mediate repression of gene expression by binding, and therefore inhibiting, the transactivation domain of E2F transcription factors, as well as by the recruitment of chromatin remodeling co-repressor complexes. Many of these interactions are through the LxCxE binding motif shared by all RB family members. All three pocket proteins form complexes with histone deacetylases, particularly HDAC1 (pRB – Luo et al., 1998; Brehm et al., 1998; Magnaghi-Jaulin et al., 1998; p107/p130 – Ferreira et al., 1998; Steigler et al., 1998). The recruitment of HDACs promotes a more condensed chromatin structure, leading to gene repression. In vivo evidence of p130/E2F4/HDAC1 and p107/E2F4/HDAC1 complexes located at the promoters of repressed genes (Ferreira et al., 2001;
Rayman et al., 2002) further supports a role for pocket proteins as co-factors for the recruitment or maintenance of chromatin modifying complexes.

*RB* family members also associate with histone methyltransferases. pRB can interact with SUV39H1 (Nielsen et al., 2001; Vandel et al., 2001), a histone methyltransferase that specifically methylates lysine 9 of histone H3, creating a binding site for HP1 and driving the formation of heterochromatin. Interaction between pRB, SUV39H1, and HP1 has been found in mouse embryonic fibroblasts (MEFs) and loss of Rb led to a loss of methylation of lysine 9 of histone H3 and HP1 binding at the *Cyclin E* promoter (Nielsen et al., 2001). A similar interaction has been found between *RB* family members and Suv-20h1 and Suv-20h2 histone methyltransferases, which methylate histone H4 at lysine 20, and are important in maintaining heterochromatin (Gonzalo et al., 2005; Isaac et al., 2006). Additionally, *RB* family members have been found to interact with BRG1 and BRGM, members of the SWI/SNF ATP-dependent chromatin remodeling complexes (Dunaief et al., 1994; Singh et al., 1995; Strober et al., 1996; Trouche et al., 1997; Dahiya et al., 2000), and this interaction may be important for inhibition of cell cycle progression. Yet, not all of *RB* family member function leads to gene repression. pRB has been found to interact with RBP2, histone H3 demethylase, in differentiating cells. Sequestration of RBP2 by pRB allows the activation of pro-differentiation genes as well as the repression of cell cycle genes (Benevolenskaya et al., 2005; Lopez-Bigas et al., 2008).

**pRB Promotes Differentiation Through Interaction with Tissue Specific Factors**

Interactions of pRB with tissue-specific co-factors have shed light on the role of pRB in cell differentiation (Figure 1-1). While pRB acts in a primarily repressive manner in regulating E2F responsive genes, pRB acts as either a co-repressor or co-activator to promote the differentiation of various tissues. In vitro models of adipogenesis using mouse embryonic lung fibroblasts have shown that pRB interacts with C/EBP transcription factors as a co-activator, increasing the ability of C/EBPs to bind DNA and activate genes promoting adipogenesis (P.L. Chen et al., 1996; Classon et al., 2000a). A similar function is seen in osteogenic differentiation, where BMP-2 induced differentiation of mouse embryonic fibroblasts (MEFs) was impaired with loss of Rb (Thomas et al., 2001). BMP2-induced MEFs were able to show early, but not late, markers of osteogenesis, implicating a role for *Rb* in late stages of osteoblast differentiation. Thomas et al. (2001) further showed that pRB promotes differentiation through interaction
with the transcription factor CBFA1/RUNX2, acting as a co-activator at osteoblast-specific promoters. pRB also works jointly with the basic helix-loop-helix transcription factor MyoD to promote differentiation of myoblasts into differentiated myotubes. MyoD activates Rb transcription during differentiation (Martelli et al., 1994; Magenta et al., 2003) and MyoD and pRB are both needed to activate MEF2, which activates the expression of late markers of muscle differentiation (Novitch et al., 1999). The pRB protein also maintains differentiated skeletal muscle in a post-mitotic state by promoting the recruitment of histone methyltransferases to silence cell cycle regulated genes (Blais et al., 2007).

Cell cycle exit and differentiation are tightly knit processes during development and pRB appears to be a key player in both processes, through interactions with E2F family members as well as with differentiation factors. Yet these two roles can be uncoupled, as detected by the study of pRB mutants. Mutant forms of pRB have been developed that do not abrogate pRB-E2F binding but are unable to induce differentiation. Additionally, pRB mutants have been developed, such as the R661W mutant, that do not bind E2Fs but still can induce differentiation in the Saos-2 osteosarcoma cell line, and cooperate with MyoD in MEFs (Sellers et al., 1998). This indicated that pRB’s role in differentiation is at least partially E2F independent. The R661W mutant of pRB was developed from a naturally reoccurring mutation that leads to low-penetrance inherited retinoblastoma, demonstrating that the differentiation function of pRB has a role in protecting against tumorigenesis (Lohmann et al., 1994; Onadim et al., 1992).

**Mouse Models Reveal Specific Functions of Rb Family Members**

Inactivation of Rb family members in mice has emphasized the temporal and tissue specific requirements for Rb family members. The constitutive inactivation of Rb in mice results in embryonic lethality by E13.5 - E15.5, and Rb-deficient embryos show increased proliferation and apoptosis within the central and peripheral nervous system, impaired erythrocyte maturation, edema, and abnormal lens development (Jacks et al., 1992; Clarke et al., 1992; E.Y. Lee et al., 1992). Yet many of these defects were later attributed to a primary placental defect. Increased proliferation of trophoblast cells within the Rb-/ placentas disrupts the proper placental architecture formation and placental transport is hindered (L. Wu et al., 2003).
The placental defect can be circumvented by either tetraploid aggregation, where cells from a wild type conceptus form only the extra-embryonic tissue and the \( Rb \) deficient cells make up the embryo proper, or by using the Cre-LoxP system using a conditional allele of \( Rb \) and the \( Meox2 \) promoter to drive Cre expression exclusively in the embryo proper (L. Wu et al., 2003; DeBruin et al., 2003b). The conditional \( RbLoxP \) allele derived by Marino et al. (2000) contains two LoxP sites surrounding exon 19 of \( Rb \), which are excised in cells expressing the Cre recombinase, leading to a non-functional \( Rb \) allele (Vooijs et al., 1998). Bypassing the placental requirement for \( Rb \) rescued the neuronal apoptosis within the central nervous system and the appearance of nucleated erythrocytes was largely reduced. These conditionally \( Rb \)-deficient embryos were able to survive until late gestation and death has been attributed to respiratory failure. However, placental rescue did not lead to complete rescue of the \( Rb \)-deficient embryo. Within the nervous system, increased proliferation and increased apoptosis was still found within the peripheral nervous system (PNS), and the severe defects in skeletal muscle development and lens development were not rescued (DeBruin et al., 2003b; MacPherson et al., 2003). Interestingly, a mutated allele of \( Rb \) comparable to the R661W mutation in low penetrance retinoblastoma, R654W, was knocked into the \( Rb \) gene in mice and created an allele deficient in binding E2F1, E2F2, and E2F3 (Sun et al., 2006). This mutant partially rescued the erythroid differentiation defect of the \( Rb \) deficient embryo, but not the cell cycle defects (Sun et al., 2006), demonstrating that the roles of pRB as regulator of cell cycle and differentiation can be unlinked. The tissue specificity of the defects in \( Rb \) deficient embryos strongly suggested that pRB is dispensable for most development but is essential for the proper development of certain tissues. \( Rb^+/- \) mice survive to adulthood, but developed pituitary and thyroid tumors (Jacks et al., 1992). Although tumors in the mice were not the same as in humans (retina versus thyroid/pituitary), this further demonstrated that in both mice and humans, the in vivo functions of pRB show tissue specificity.

Mutant mouse models, in which either \( p107 \) or \( p130 \) was inactivated, were developed by two different groups (\( p107 \) – M.H. Lee et al., 1996; LeCouter et al., 1998a; \( p130 \) - Cobrinik et al., 1996; LeCouter et al., 1998b). \( p107^-\) or \( p130^-\)-deficient mice are normal and fertile on a mixed (129Sv x C57BL/6) background, but develop strong phenotypes on a mixed (129Sv x C57BL/6) background enriched with Balb/c. Loss of \( p130 \) on this enriched Balb/c background leads to embryonic lethality by E13 with defects in neuronal, muscle, and heart development (LeCouter et al., 1998b). Mice deficient in
on this mixed Balb/c background survive, but show impaired growth and develop myeloproliferative disorders (LeCouter et al., 1998a). However, these phenotypes were strain-specific, because when these mutant animals are crossed to a C57BL/6 background, the developmental phenotypes are lost, and p107-deficient or p130-deficient mice show no major phenotypes, agreeing with the earlier models developed by Cobrinik (1996) and M.H. Lee (1996). What the potential strain-specific modifiers of p130 and/or p107 function has yet to be resolved.

p107 and p130 doubly deficient mice die at birth with defects in bone development (Cobrinik et al., 1996). Combining loss of p107 with Rb deficiency exacerbates the Rb phenotype, and there was an increase in apoptosis within the fetal liver and the central nervous system (M.H. Lee et al., 1996), the same tissues that show defects with loss of Rb alone. A more recent model of combined p107 deficiency with conditional loss of Rb led to earlier lethality, with embryos dying between E13.5-E14.5, with increased proliferation and/or apoptosis within the CNS, liver, muscle, and erythroid cells, and previously unseen defects in heart development (Berman et al., 2009). Thus, the loss of two Rb family members show only tissue-specific defects during development, even though the pocket proteins are thought to be key players in regulating the cell cycle in most, if not all, tissues. Using tetraploid aggregation, embryos with wild type placenta but deficient in all three Rb family members within the embryo proper, only survived until embryonic day E9 (Wirt et al., 2010). Interestingly, these embryos were still able to form many of the proper developmental structures such as the neural tube and the cardiac chambers, indicating that Rb family members are not required in all tissues for early embryonic development.

Tissue specific phenotypes or apparent dispensability appear to be common outcomes from inactivating cell cycle regulators. Mutant embryos are viable despite the loss of Cdk (e.g. Cdk4 or Cdk6), Cyclins (e.g. CcnE1 or CcnD1), and Cdk inhibitors (e.g. Ink4a/Arf or p15Ink4b). Triple knockout of all three genes encoding Cyclin D proteins (CcnD1-3) only leads to embryonic lethality at E16 with hematopoietic defects (Kozar et al., 2004). Similarly, mice deficient in Cdk2 and Cdk6 are viable with hematopoietic defects and sterility (Malumbres et al., 2004). Additionally, mice deficient in Cdk2, Cdk4, and Cdk6 can survive until E12.5-E13.5 and die primarily due to hematopoietic defects while Cdc2a-deficient (Cdk1) embryos cannot be found as early as E1.5, indicating that while the mitotic Cyclins/Cdks are required for early development, many of the G1 Cyclins and Cdks are dispensable (Santamaria et al., 2007). These
results highlight that regulation of the cell cycle during development differs between tissues and that where and when cell cycle regulators are expressed may play a large role in their function.

Mouse Models of Retinoblastoma

The fact that \(Rb\) LOH did not lead to tumorigenesis in the \(Rb^{+/-}\) mouse retina led a number of laboratories to search for what could be suppressing retinoblastoma formation. This led to the development of various mouse models of retinoblastoma. While \(Rb^{+/-};p107^{-/-}\) mice only developed retinal dysplasia (M.H. Lee et al., 1996), formation of retinoblastoma appears to require the loss of at least one other pocket protein and potentially the p53 pathway. The first models of retinoblastoma were observed in transgenic mice expressing the T-antigen of the SV-40 virus under control of the \(\beta\)-luteinizing hormone promoter, which leads to loss of function of both the \(Rb\) and p53 pathways (Windle et al., 1990). This was followed by the generation of chimeric mice using ES cells deficient for \(p107\) and \(Rb\) (Robanus-Maandag et al., 1998), which also developed retinoblastoma, indicating that \(p107\) may compensate for \(Rb\) loss in the mouse retina and protect against tumor formation. Knockout models of retinoblastoma were generated by conditionally inactivating an \(Rb^{LoxP}\) allele in a \(p107\) or \(p130\) deficient background using \(Chx10-Cre\), \(Nestin-Cre\), or \(Pax6-Cre\), all of which lead to excision of the \(Rb\) locus within retinal progenitors (MacPherson et al., 2004, J. Zhang et al., 2004a, D. Chen et al., 2004). While loss of \(p107\) or \(p130\) did not lead to tumor formation in mice, the retinoblastoma models demonstrate that \(p107\) and \(p130\) have tumor suppressor functions in the retina in the context of \(Rb\) loss. The phenotype of retinoblastoma with loss of \(Rb\) and \(p107\) is not equivalent to loss of \(Rb\) and \(p130\); animals deficient in \(Rb\) and \(p107\) in the retina develop a unilateral retinoblastoma with partial penetrance while animals deficient in \(Rb\) and \(p130\) develop bilateral retinoblastoma with 100% penetrance (Dannenberg et al., 2004; MacPherson et al., 2004; D. Chen et al., 2004). While loss of \(Rb\) family member function is necessary for the initiation of retinoblastoma, loss of the p53 pathway has been implicated as one of the secondary hits needed for progression in mice. Additional loss of \(p53\) in \(Chx10^{-/-};Rb^{Lox/-};p107^{-/-}\) retina increases both the penetrance and severity of the retinoblastoma phenotype (J. Zhang et al., 2004a). Although no \(p53\) mutation has been found in human retinoblastoma, amplification of \(MDMX\) or \(MDM2\), encoding inhibitors of \(p53\), is prevalent (Laurie et al., 2006) and may contribute to human retinoblastoma progression.
*Rb* Family Expression During Development and Differentiation

The *Rb* family members are highly expressed in distinct and overlapping tissues during development. In situ analysis (Jiang et al., 1997) has shown that *Rb* expression is limited to the developing nervous system, fetal liver, muscle, and to the retina and lens of the developing eye with highest expression between E12.5 and E14.5. This tissue-specific expression of *Rb* correlates well with the known developmental defects of the *Rb*-deficient embryo, lending support to the idea that regulating *Rb* expression is important for proper embryonic development. *p107* has a broader pattern of mRNA expression, which overlaps with *Rb* in the CNS and fetal liver, and is also found in the heart, lungs, kidney, and gut at various embryonic ages (Jiang et al., 1997). In the CNS, *p107* expression is turned on at earlier embryonic time points (E10.5) within the proliferative ventricular zone and its expression declines by E15.5 (Jiang et al. 1997; Burkhart et al., 2008). This pattern of decreased expression of *p107* with increasing maturation of the nervous system is consistent with the increase in *p107* expression as cells enter S phase and supports a role for p107 in cycling cells during development. In contrast, *p130* shows a ubiquitous and diffuse expression pattern within the developing embryo (Jiang et al., 1997; G. Chen et al., 1996). Tissue specificity appears to play an important role in distinguishing functions of the pocket proteins in vivo.

While the post-translational regulation of pRB has been well studied (e.g., phosphorylation), much less is understood about the regulation of *Rb* expression. Tissue culture experiments have shown that the levels of *Rb* at the protein and mRNA level increase as cells are induced to differentiate. The P19 embryonal carcinoma cell can be induced to differentiate towards a neuronal fate with the addition of all-trans retinoic acid (ATRA) or towards mesodermal and endodermal fates with the addition of dimethyl sulfoxide (DMSO) (McBurney, 1993). When P19 cells were treated with ATRA, the levels of pRB protein (Gill et al., 1998) and *Rb* mRNA (Slack et al., 1993) increased as cells became more differentiated. The increase was also seen with DMSO induced differentiation, but to a lesser extent. Levels of p130 also increase during differentiation of P19 cells, while *p107* levels increase but then fall off as cells become post-mitotic (Gill et al., 1998). The increase of *Rb* expression with differentiation is not limited to P19 cells. Protein and mRNA levels of *Rb* increase as myoblasts differentiate into myotubes (Kiess et al., 1995; Coppola et al., 1990) and as mouse erythroleukemia cells (MEL) are induced to express mature
erythrocytic markers with HMBS (Coppola et al., 1990). The increase in RB mRNA is also seen in 1,25-dihydroxyvitamin D₃ induced differentiation of human leukemia HL60 cells toward a monocytic lineage (white blood cell) and this correlates with the interaction of pRB with C/EBP transcription factors (Ji and Studzinski, 2004). Thus, Rb levels increase with differentiation of neuronal, myogenic, and hematopoietic lineages, the three lineages in which Rb-deficient embryos show strong phenotypes.

**Regulation of Rb Expression**

The study of low penetrance retinoblastomas uncovered an important regulatory region of the RB gene. Point mutations in potential Sp1 and ATF binding sites were uncovered upstream of the RB start site and these mutations correlated with loss of RB expression within tumor cells from low penetrance cases of retinoblastoma patients (Sakai et al., 1991a; Fujita et al., 1999; Taylor et al., 2007). These sites are located between -207 and -179 upstream of the RB start site (-198bp to -85bp in mouse) and are within a highly GC rich region shown to be the essential promoter region necessary for RB expression (Figure 1-3) (Gill et al., 1994). This region contains a complex 26 base pair element containing Sp1 and ATF (overlaps with a site for Ets binding) sites, and an adjacent E2F binding site, that is 100% conserved between mouse and human (Zacksenhaus et al., 1993; Gill et al., 1994). Experiments using Rb promoter sequences attached to a luciferase reporter showed that loss of the E2F site leads to an increase in reporter activity in certain cell lines (Ohtani-Fujita et al., 1994), implicating the E2F site as a repressive site. Over-expression studies of pRB in cell culture demonstrated that introduction of pRB decreases activity from Rb promoter-CAT constructs (Hamel et al., 1992; Shan et al., 1994), and that pRB was repressing its own promoter, potentially through the E2F site, via an auto-regulatory loop. Furthermore, in retinoblastoma tumor samples where RB LOH has occurred, there were high levels of transcript derived from the mutant allele, which was barely present in normal tissue from the same patients (Dunn et al., 1988). It was postulated that in normal tissue functional pRB represses transcription of the RB mutant allele, but this repression is lost when LOH occurs, and mutant transcript is produced. These results made pRB a good candidate for being the repressor of the RB promoter.

The importance of RB expression, both in differentiation and in preventing tumorigenesis, suggests that regulation of the Rb promoter in an E2F dependent manner is an additional way by which differentiation and cell cycle exit are linked during development. To determine how the Rb promoter is
**Figure 1-3**: *Rb1* promoter sequence is highly conserved between mouse and human. The regulatory region 235 bp upstream of the ATG of *Rb1* is 80% homologous between mouse and human and contains a conserved cassette containing an SP1/Ets, ATF, and E2F site. The start site for the mouse pRB protein is boxed.
regulated in vivo, our laboratory generated a mouse model expressing a LacZ reporter transgene driven by the mouse Rb promoter (includes 4.3kb upstream of the Rb translational start site including the minimal promoter) designated RbP(WT)-LacZ (Figure 1-4) (Agromayor et al., 2006). Three independent transgenic lines for the RbP(WT)-LacZ reporter showed a neuronal specific pattern of Rb promoter activity, indicating that this promoter region was important for Rb expression within the central and peripheral nervous systems. The neuronal expression pattern correlates strongly with Rb expression seen by in situ analysis, and only a subset of neurons showed LacZ activity within the developing cortex, retina, trigeminal ganglion and dorsal root ganglia. The extent of LacZ activity within the nervous system increased with developmental time indicating that regulation of Rb during development is not static (Agromayor et al., 2006). This expression pattern agrees with Rb promoter–LacZ transgenics made independently (Jiang et al., 2001) showing exclusively neuronal expression. The additional regulatory elements needed for Rb expression within the muscle and liver are not located within this 4.3kb region, but instead appear to lie further upstream (Burkhart et al., 2010a).

Since previous evidence from low penetrance retinoblastomas had shown that a critical Sp1 site was important for activation of the Rb promoter in human retinoblastoma, our laboratory generated mice with a mutant Rb promoter-LacZ transgene bearing a triple substitution (AAA) within the Sp1 and the overlapping Ets site (Agromayor et al., 2006). RbP(Sp1/EtsAAA)-LacZ embryos had very low LacZ activity within the developing nervous system, supporting the notion that normally this Sp1/Ets site is needed for activation of the Rb promoter in vivo (Figure 1-5). When our laboratory made a similar AAA mutation within the E2F site, RbP(E2FAAA)-LacZ embryos displayed increased LacZ activity restricted mostly to the nervous system, although an increase in activity was also seen in some muscle tissue (Figure 1-5). The increase in LacZ activity in RbP(E2FAAA)-LacZ embryos was particularly strong within the developing cortex. While RbP(WT)-LacZ embryos showed moderate LacZ activity restricted to the lateral regions of the cortex, RbP(E2FAAA)-LacZ embryos showed strong LacZ activity at the midline of the cortex. In contrast, RbP(Sp1/EtsAAA)-LacZ embryos had extremely low LacZ activity within the cortex and a low level of LacZ activity was observed in other neuronal regions. A similar effect was seen within the retina. In the adult RbP(WT)-LacZ retina, LacZ activity is restricted to the ganglionic and inner nuclear layers and not the outer nuclear layer, but in the RbP(E2FAAA)-LacZ retina, LacZ activity is
Figure 1-4: Structure of the RbP-LacZ transgene.
The RbP-LacZ transgene consists of a 4.3Kb region upstream of the Rb start site, including the cluster of transcription factor binding sites (green), driving a nuclear LacZ reporter. Loss of the E2F binding site in the RbP(E2FAAA)-LacZ transgenic mouse led to an increase in reporter activity in vivo, implicating this as a repressive site. Therefore, it is postulated that Rb family members may repress Rb expression, potentially through this E2F site.

Figure 1-5: Patterns of LacZ staining in Rb promoter mutant reporter mice.

Panel A lists the locations within the nervous system where LacZ activity is detected in the various Rb promoter transgenic lines using Xgal staining. Disruption of the potential Sp1/Ets site within the Rb promoter led to a loss of LacZ activity within the developing neocortex (A and B), as well as a reduction of LacZ activity within the adult retina, indicating that it is an activating site. Disruption of the potential E2F binding site led to a de-repression of the RbP-LacZ reporter within the midline of the neocortex (A and B) as well as in the ONL of the adult retina (A), indicating that this site is necessary for repression of Rb expression. Similar results were seen in multiple transgenic lines. (A) INL = inner nuclear layer; GCL = ganglionic cell layer; ONL = outer nuclear layer; (B) A = anterior; P = posterior.

Adapted from Agromayor et al., 2006
found in all three retinal layers at very high levels. Interestingly, the increase in LacZ activity within the RbP(E2FAAA)-LacZ retina at E16.5 was minimal, indicating that de-repression of the Rb promoter is a dynamic process. Thus, this repressive E2F site in the Rb promoter regulates Rb expression at specific developmental times and in specific neuronal compartments during development.

**Specific Aims**

The extensive deregulation of the Rb promoter within the nervous system with loss of the E2F site demonstrated that E2F regulation of the Rb promoter is a tightly regulated process, which is spatially and temporally dynamic during development (Agromayor et al., 2006). The increased activity of the RbP(E2FAAA)-LacZ reporter in vivo reveals that this E2F site is needed for repression of the Rb promoter, implicating pocket protein regulation. Although many tissue culture studies have postulated that pRB is a repressor of its own promoter, it is not inconceivable for all three pocket proteins to be potential regulators of Rb expression. The potential for Rb family members to regulate expression of other family members has precedent; pRB has been found to regulate p107 expression in MEFs (Schneider et al., 1994; Hurford et al., 1997). Additionally, both E2F1 and E2F4 were shown by gel shift using cortical nuclear extracts and chromatin immunoprecipitation from embryonic cortex to be able to associate with the Rb promoter (Agromayor et al., 2006). This opens up the possibility for RB family members to directly regulate the Rb expression, potentially through interactions with E2Fs. Therefore, the first question of this study was to ask if Rb family members are repressors of the Rb promoter in vivo and which pocket protein(s) are responsible for this repression (Aim 1, Chapter 2).

To test if Rb family members are repressors of the Rb promoter in vivo and to understand how expression of Rb functions in proper neuronal development, the wild type RbP-LacZ transgenic mouse line, T157, developed in by Agromayor et al (2006) was crossed to mice deficient in individual or combinations of Rb family members. The expectation was that if Rb family members are playing a repressive function to control Rb expression, then loss of one or more of the Rb family members should lead to an increase in activity from the RbP-LacZ transgene (Figure 1-4). Since the localization of RbP-LacZ activity appears to be temporally and spatially controlled in vivo, it was essential to perform these experiments within the mouse embryo where this specificity is seen. Analysis was focused on the developing cortex, because it displayed the most consistent LacZ activity between the different
RbP(WT)-LacZ mice generated, and showed dramatic de-regulation of LacZ activity in the RbP(E2FAAA)-LacZ embryo.

The Rb family members regulate a large number of genes through interactions with E2Fs and chromatin modifiers, and so changes in RbP-LacZ activity seen with Rb family loss could be due to indirect effects of the various Rb family deficiencies. De-regulation of E2F activity may affect cellular processes such as proliferation, apoptosis, and neuronal development that could lead to changes in RbP-LacZ activity within the embryo that are independent of direct changes at the Rb promoter. Therefore, the second question of this study was to ask whether Rb family members regulate the Rb promoter by direct binding of the Rb promoter and/or through indirect effects of Rb family deficiency (Aim 2, Chapter 3). To differentiate between direct regulation by Rb family members at the Rb promoter and secondary effects due to developmental changes, a combination of chromatin immunoprecipitation, immunohistochemistry, and gene expression analysis was performed on embryos deficient in various Rb family members.

As the cortex develops, precursor cells generate a variety of neuronal and glial cell types, in a temporally and spatially specific manner to create a complex laminar structure that becomes the adult cerebrum. One possibility is that loss of Rb family members may disrupt the process of cortex formation, and thereby lead to non-cell autonomous changes in RbP-LacZ activity in the cortex, a variation of the indirect mechanisms explored above. Therefore, to distinguish between non-cell autonomous effects and cell autonomous changes in RbP-LacZ activity with Rb family deficiency, we developed primary cortical cultures from mutant embryos to allow for single cell analysis (Aim 3, Chapter 4). Primary cortical culture allows us to determine, on a cell-by-cell basis, whether RbP-LacZ activity changes in frequency and/or intensity with Rb family deficiency, indicating whether changes in RbP-LacZ activity are due to cell autonomous effects. Another important question to be answered using primary cortical culture is what cell types exhibit RbP-LacZ activity. The identity of the cell types with RbP-LacZ activity allows us to discern where and when Rb family members may be regulating Rb expression during neuronal development.
Chapter 2: Rbp-LacZ Activity in Embryos Lacking Various Rb Family Members

In Situ Analysis of Rbp-LacZ Activity in Embryos at E14.5

Analysis of embryos deficient in p107 or p130
Analysis of embryos deficient in p107 and p130
Analysis of conditional Rb-deficient embryos
  Using Meox2-Cre on a Mixed genetic background
    (129/Ola x FVB) x C57BL/6
  Using Meox2-Cre on an inbred C57BL/6 background
  Excision using Meox2-Cre versus NestinCre models
  Neuronal specific loss of Rb

In Situ Analysis of Rbp-LacZ Activity in Isolated Cortex

Analysis of the E14.5 and adult cortex
Analysis of the p130-deficient cortex
Analysis of the p107 and p130-deficient cortex
Analysis of the conditional Rb-deficient cortex

Summary

In Situ Analysis of Rbp-LacZ Activity in Embryos at E14.5

To determine if Rb family members are repressors of the Rb promoter in vivo, the T157 Rbp(WT)-LacZ line, developed by Agromayor et al. (2006), was crossed to mouse lines deficient in p107, p130, or Rb. Mid-gestational embryos were generated, which were deficient in Rb, p107 or p130 or both p107 and p130 and analyzed for Rbp-LacZ activity. Rbp-LacZ activity was monitored by Xgal staining over night, followed by fixation before microscopic/photographic analysis. Changes in LacZ activity were determined by the following criteria: observation of a change in the localization of Xgal staining within the neocortex, observation of a change in the strength of Xgal staining within the neocortex, observation of a change in the pattern of Xgal staining in other neuronal and non-neuronal tissues. Comparative analysis of embryos was done by visual inspection of embryo images from front, top, side, and rear viewpoints. The expectation was that if an Rb family member is a repressor of the Rb promoter, then a loss-of-function mutation for this family member should result in an increase in Rbp-LacZ reporter activity.

Analysis of Rbp-LacZ reporter in either p107- or p130-deficient E14.5 embryos

T157;p107+/- mice were mated to p107+/- mice for timed pregnancies and embryos were harvested for whole mount Xgal staining at E14.5. When comparing T157;p107+/- embryos to
Figure 2-1: Loss of p107 does not deregulate RbP-LacZ activity at E14.5.
Embryos from a T157;p107 litter were stained with Xgal solution overnight. LacZ activity was visualized as blue staining in the presence of Xgal. There is no dramatic change of RbP-LacZ activity seen with loss of p107.

Figure 2-2: Loss of p130 does not deregulate RbP-LacZ activity at E14.5.
Embryos from a T157;p130 litter were stained with Xgal solution overnight. Loss of p130 did not change RbP-LacZ activity within the embryo.
T157;p107-/ embryos, there was no consistent change in the intensity or the localization of LacZ activity (Figure 2-1). This was seen in two separate dissections (T157;p107+/+, n=4; T157;p107-/-, n=3). Thus, loss of p107 alone is not enough to deregulate RbP-LacZ activity in the developing embryo.

Similarly, T157;p130+/− mice were crossed to p130+/− mice and embryos were harvested and analyzed for whole mount Xgal staining at E14.5. In one litter, loss of p130 led to a decrease in LacZ activity, but this decrease was not seen in a subsequent dissection (for both litters, T157;p130+/+, n=3; T157;p130−/−, n=3) (Figure 2-2). Therefore, loss of p130 alone does not deregulate RbP-LacZ activity in a consistent manner in the developing embryo.

Analysis of RbP-LacZ reporter with dual loss of p107 and p130 in E14.5 embryos

Loss of p107 or p130 alone has been shown to have only small effects on the cell cycle as well as E2F target gene expression in culture (Hurford et al.,, 1997). Additionally, on a C57BL/6 x 129Sv background, p107-deficient and p130-deficient mice are grossly normal and live normal life spans (Cobrinik et al., 1996; M.H. Lee et al., 1996). Yet, when both p107 and p130 are lost, p107−/−;p130−/− embryos die at birth, indicating that the proteins have overlapping functions (Cobrinik et al., 1996). Therefore, we analyzed RbP-LacZ expression in T157 mice deficient in both p107 and p130. This three factor cross was performed on a p130−/− background to increase the efficiency of recovering T157;p107−/−;p130−/− embryos. This strategy was chosen, because no consistent changes in RbP-LacZ activity were seen with loss of p130, and p107+/−;p130−/− animals were able to breed efficiently, while p107−/−;p130+/− females were poor breeders due to defects in the reproductive tract (imperforate vagina). T157;p107+/−;p130−/− mice were crossed to p107+/−;p130−/− mice for timed pregnancies, and T157;p107+/−;p130−/− and T157;p107+/−;p130−/− embryos were harvested and analyzed by whole mount Xgal staining at E14.5. We observed decreased activity of the RbP-LacZ transgene within the nervous system, particularly the cortex, with loss of both p107 and p130 (Figure 2-3). There was no deregulation of RbP-LacZ activity observed in other tissues. This decrease was consistent, as it occurred in 11 out of 11 T157;p107−/−;p130−/− embryos from 8 different dissections (Table 2-1) at E14.5 and E15.5. Surprisingly, this result implies that p107 and p130, known repressors of E2F-dependent gene expression, are needed for activation of the Rb promoter.
Figure 2-3: Decreased Rb promoter activity with p107 and p130 deficiency at E14.5.

Embryos from a representative T157; p107+/-;p130-/- x p107+/-;p130-/- cross were stained with Xgal solution overnight. There is a decrease in intensity of Xgal staining within the lateral regions of the neocortex (filled arrow) in T157; p107-/-;p130-/- embryos (C and D) compared to in litter controls (A and B). T157; p107-/-;p130-/- embryos showed limb defects as previously reported (unfilled arrow).
Table 2-1: Decrease in RbP-LacZ activity with dual loss of p107 and p130 at E14.5 and E15.5

Eight different litters, represented by female number, were analyzed to determine if loss of p107 and p130 led to deregulation of RbP-LacZ activity. All crosses were done on a p130 deficient background between T157;p107+/-;p130-/- and p107+/-;p130-/- mice. A decrease in RbP-LacZ activity was seen in all 11 T157;p107-/-;p130-/- embryos compared to in litter controls.
Analysis of RbP-LacZ reporter in Rb conditional knockout E14.5 embryos

Analysis of RbP-LacZ reporter in Meox2Cre;RbLoxP/LoxP embryos on a mixed genetic background

Constitutive loss of Rb leads to a placental defect and subsequent embryonic lethality between E13.5-E15.5, and mutant embryos have severe apoptosis in the central and peripheral nervous systems (Clarke et al., 1992; Jacks et al., 1992; E.Y. Lee et al., 1992). Since RbP-LacZ (T157) is expressed primarily in the nervous system, and strong LacZ activity only is seen starting at approximately E13.5. Therefore, the constitutive Rb knockout is not an ideal model for analyzing the effect of Rb loss on RbP-LacZ. Therefore, we used a conditional knockout model to avoid the placental requirement for Rb that is responsible for the mid-gestational lethality of the constitutive Rb knockout. We crossed RbLoxP/LoxP mice with LoxP sites surrounding Rb exon 19 (maintained on a 129/OLA x FVB background) (Marino et al., 2000) to either T157 mice (on a C57BL/6 background) or mice with Cre recombinase knocked into the Meox2 locus (on a C57BL/6 background) (Tallquist and Soriano, 2000). Meox2-Cre specifies Cre expression within the epiblast that is destined to become the embryo proper, but does not lead to Cre expression within the extra-embryonic tissue. By maintaining wild type Rb within the extra-embryonic tissue, the placental defect due to Rb deficiency is rescued and conditional Rb-deficient embryos can survive until birth (DeBruin et al., 2003b). Meox2-Cre;RbLoxP/+ mice were crossed to T157;RbLoxP/LoxP mice for timed pregnancies and embryos were harvested and analyzed for LacZ activity by whole mount Xgal staining at E14.5. In six separate dissections, Rb-deficient embryos (T157;Meox2Cre;RbLoxP/X, where X denotes the excised LoxP allele inherited through the germline) showed a consistent increase in RbP-LacZ activity within the nervous system stem relative to that seen in wild type (T157;RbLoxP+/+) embryos, as determined by an increase in the intensity of Xgal staining as well as an increase in the broadness of Xgal staining within the lateral regions of the neocortex. There was no deregulation of RbP-LacZ activity seen in other tissues. A consistent increase was also seen within the mid-brain and spinal cord, but analysis was precluded by edema in Rb-deficient embryos.

Analysis of RbP-LacZ reporter in Meox2-Cre;RbLoxP/LoxP embryos on an inbred C57BL/6 background

Although the increase in RbP-LacZ activity was consistent using Meox2-Cre to drive loss of Rb, there was variation in the magnitude of the increase in LacZ activity and in the health of the embryos. This variation could be due to the mixed genetic background (129/Ola x FVB x C57BL/6) of the resultant
Figure 2-4: De-repression of the $Rb$ Promoter with conditional loss of $Rb$ at E14.5 on C57BL/6xFVB background

A representative set of embryos from a cross between $\text{Meox2Cre;RbLoxP/+}$ and $\text{T157;RbLoxP/LoxP}$ mice on a mixed C57BL/6xFVB background were stained overnight in Xgal solution. An increase in the intensity and broadness of $RbP$-LacZ activity can be seen in $Rb$-deficient ($\text{T157;Meox2Cre;RbLoxP/X}$) embryos within the neocortex (C and D) compared to embryos wild type (A) or heterozygous (B) for $Rb$ (filled arrows).
Table 2-2: Increase in \( RbP\)-LacZ activity with conditional loss of \( Rb \) using \( Meox2Cre \) on a C57BL/6xFVB background. Six different litters, represented by female number, were analyzed to determine if conditional loss of \( Rb \) in the embryo proper led to deregulation of \( RbP\)-LacZ activity. Crosses were done between \( Meox2Cre;RbLoxP/+ \) and \( T157;RbLoxP/LoxP \) mice with either the source of Cre recombinase being maternal or paternal. One excised allele is passed down through the germline through the \( Meox2Cre;RbLoxP/+ \) parent and is represented by an X. All comparisons were made to in litter controls.

<table>
<thead>
<tr>
<th>Female</th>
<th>Cre Source</th>
<th># Embryos</th>
<th>( T157; RbLoxP/+ )</th>
<th>( T157; Meox2Cre; RbLoxP/X )</th>
<th>( T157; Meox2Cre; RbLoxP/X )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1513</td>
<td>Mat</td>
<td>8</td>
<td>3</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>T998</td>
<td>Mat</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>A1530</td>
<td>Mat</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>A1264</td>
<td>Pat</td>
<td>8</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>A1335</td>
<td>Pat</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>A1341</td>
<td>Pat</td>
<td>8</td>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>42</td>
<td>24</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2-2: Increase in \( RbP\)-LacZ activity with conditional loss of \( Rb \) using \( Meox2Cre \) on a C57BL/6xFVB background.
Figure 2-5: Increased RbP-LacZ activity with conditional Rb loss at E14.5 on a C57BL/6 background. Embryos from a cross of Meox2Cre;RbLoxP/+ x T157;RbLoxP/LoxP on a C57BL/6 background were stained overnight in Xgal solution. An increase in RbP-LacZ activity can be seen in the Rb-deficient (T157;Meox2Cre;RbLoxP/X) neocortex (column B) compared to the littermate control (column A) (Filled arrows). The top and bottom images are of the same embryo to demonstrate how the increase in RbP-LacZ activity is not throughout the whole cortex but restricted laterally. The T157;Meox2Cre;RbLoxP/X embryo has a slight curvature due to edema, which was present in 100% of Meox2Cre;RbLoxP/X embryos at this time point.
Table 2-3: Increase in RbP-LacZ activity with conditional loss of Rb using Meox2Cre on a C57BL/6 background. Six different litters, from crosses between Meox2Cre;RbLoxP/+ and T157;RbLoxP/LoxP mice, were analyzed to determine if conditional loss of Rb in the embryo proper led to deregulation of RbP-LacZ activity. An increase in RbP-LacZ activity was seen in five of seven embryos analyzed.
embryos. One obvious example of this variation was differences in eye pigmentation of embryos within the same litter, ranging from no eye pigmentation (from the 129/Ola x FVB background) to black eye pigmentation (from the C57BL/6 background). Since the phenotypes resulting from loss of various Rb family members are modified by genetic background (LeCouter et al., 1998a; LeCouter et al., 1998b; Leung et al., 2004), RbLoxP/LoxP mice were backcrossed onto the C57BL/6 background. After 6-10 backcrossed generations, these RbLoxP/LoxP (B6) mice were then mated to either the Meox2-Cre or T157 lines, and experiments were repeated to test whether conditional loss of Rb changed RbP-LacZ activity on an inbred C57BL/6 background. Similar to the cross on a mixed genetic background, conditional loss of Rb led consistently to an increase in RbP-LacZ activity within the nervous system (Figure 2-5 and Table 2-3), implicating pRB as a repressor of its own promoter, a theory consistent with previous data that the E2F site in the Rb promoter is a repressive site.

Excision efficiency driven by Meox2-Cre vs. Nestin-Cre in the CNS

We chose a Meox2-Cre conditional model because it leads to loss of Rb in all tissues of the embryo proper, allowing us to determine if Rb family deficiency would lead to changes in RbP-LacZ activity in either neuronal or non-neuronal tissues. To estimate the excision efficiency of LoxP flanked alleles driven by Meox2-Cre within the embryo, we crossed Meox2-Cre mice to ROSA26LoxP-STOP-LoxP reporter mice (R26R) developed by Soriano (1999). The R26R line contains a STOP sequence upstream of a LacZ reporter that is flanked by two LoxP sites. When Cre recombinase is expressed within the cell, it excises the STOP sequence and expression of LacZ is then driven by the ROSA26 locus, which is ubiquitously expressed. Whole mount Xgal staining of Meox2Cre;R26R embryos showed efficient excision by the Meox2Cre within most of the embryo, except for the nervous system (Figure 2-6), which is the predominant tissue with RbP-LacZ activity and thus, of highest interest. Therefore, we decided to evaluate excision efficiency in R26R embryos using a neuronal specific Nestin-Cre transgenic line to delete LoxP-STOP-LoxP conditionally. Whole mount Xgal staining of NestinCre;R26R embryos showed highly specific excision at the ROSA26 locus within the nervous system (Figure 2-6). In addition, use of the neuronal specific Nestin-Cre would avoid any secondary deleterious effects of Rb deficiency in other tissues that may occur in the Meox2-Cre conditional knockout.
Figure 2-6: Efficient activation of the ROSALox-STOP-Lox reporter in the CNS by Nestin-Cre at E16.5. Mice containing the ROSALox-STOP-Lox cassette (R26R) which measures Cre-mediated excision in the presence of Xgal, were crossed to Meox2Cre and Nestin-Cre mice to generate E16.5 embryos. The Meox2Cre;R26R embryo (A) shows strong blue staining within almost all tissues except the CNS that only showed patchy staining. The Nestin-Cre;R26R embryo (B) shows activation exclusively within the nervous system. Intestinal staining within Nestin-Cre;R26R embryos was not specific and was seen in R26R littermates that did not contain the Nestin-Cre transgene. Micro-dissected brains were also stained with Xgal at E14.5 with similar results. Xgal staining within the intestines was seen in both control and Cre positive embryos, and was therefore determined to be non-specific.
Figure 2-7: Verifying loss of Rb through Cre mediated excision of the RbLoxP locus.

The DNA from the inner yolk sacs (iys) and midbrains (mb) of embryo litters was extracted and used for genotype analysis by PCR. Each letter represents a different embryo in the litter. Panel (A) shows the presence of the Nestin-Cre transgene as well as the sex (Zfy) for each embryo. Panel (B) shows the excision of the LoxP site in embryos that express Nestin-Cre using primers floxing the LoxP site (Rb18L, Rb19EL, Rb212; see Methods section). Embryo I contains two LoxP alleles within the inner yolk sac, which becomes one excised band in the midbrain. Embryo G shows one LoxP allele and one excised allele, presumed to be inherited through the germline, in the inner yolk sac, while the midbrain sample shows excision of the remaining allele. The first lane in each gel contains 100bp ladder. DNA samples from the parents were used as controls. Amplification of the wild type Dp1 locus was performed concurrently as a loading control.
Analysis of RbP-LacZ reporter with neuronal specific loss of Rb in E14.5 embryos

The Nestin-Cre transgene contains the second intron of the rat Nestin gene, which contains a neuronal specific enhancer upstream of Cre recombinase (Zimmerman et al., 1994), that drives Nestin-Cre activity by E11 (Graus-Porta et al., 2001). Using PCR to amplify short genomic segments with primers lying outside of the LoxP flanked exon 19, we observed efficient excision of exon 19 in midbrain tissue from NestinCre;RbLoxP/LoxP embryos, but no excision within the inner yolk sacs, as expected (Figure 2-7). Interestingly, sporadic excision of the RbLoxP allele was seen occasionally within the inner yolk sac in embryos irrespective of the presence of the Nestin-Cre transgene, which likely is a germline event that was also detected in adult animals. Although the Nestin promoter element used in these Cre-expressing lines is thought to be neuronal specific, it has been reported that Nestin-Cre is expressed within the germline, and can lead to excision in the germline (Bates et al., 1999). Therefore, both T157;NestinCre;RbLoxP/X and T157;NestinCre;RbLoxP/LoxP embryos were designated as Rb-deficient within the nervous system.

NestinCre;RbLoxP/+ mice were crossed to T157;RbLoxP/LoxP mice for timed pregnancies and the resultant wild type (T157;RbLoxP/+ or T157;RbLoxP/LoxP) and Rb-deficient embryos were analyzed for whole mount LacZ activity with Xgal staining at E14.5. Midbrains were removed after Xgal staining and used to confirm excision of the RbLoxP locus by PCR analysis. In 14 of 21 Nestin-Cre;Rb-deficient embryos, there was an increase in RbP-LacZ activity within the developing cortex and in the spinal cord (Figure 2-8 and Table 2-4). Conditional loss of Rb specifically in the nervous system led to an increase in activity of the RbP-LacZ reporter, again implicating pRB as a repressor of its own promoter within the nervous system.

In Situ Analysis of RbP-LacZ Activity in the Isolated Cortex

RbP-LacZ activity in the wild type T157 cortex at E14.5 and in the adult

Since we knew from our laboratory’s previous work that loss of the E2F site within the RbP-LacZ reporter leads to de-repression of LacZ activity within the cortex in a spatially-specific manner (Agromayor et al., 2006), we wanted to see whether loss of Rb family members alters RbP-LacZ activity in a spatially restricted manner. To test this, we decided to Xgal stain microdissected cortices from wild type and various Rb family deficient embryos containing the RbP-LacZ reporter. The mouse neocortex begins to
Figure 2-8: Increased RbP-LacZ activity with conditional Rb loss in the CNS at E14.5. Embryos from a representative T157;RbLoxP/LoxP x Nestin-Cre;RbLoxP/+ cross were stained with Xgal solution overnight. There is an increase in intensity and the broadness of Xgal staining within the cortex (filled arrows) of Rb deficient embryos (C and D) compared to in litter controls (A and B).
Table 2-4: Increase in RbP-LacZ activity with conditional loss of Rb in the CNS using Nestin-Cre.

Eleven different litters were analyzed to determine if conditional loss of Rb in the CNS led to deregulation of RbP-LacZ activity. Crosses were done between Nestin-Cre;RbLoxP/+ and T157;RbLoxP/LoxP mice, with the source of Cre inherited maternally or paternally. The * indicates that there was an additional embryo within that litter which showed gross developmental defects and therefore was not used for analysis.
develop at E11 and continues to develop post-natally. A germinal zone of proliferative cells gives rise to a laminar structure consisting of diverse neuronal subtypes (reviewed in Dehay and Kennedy, 2007) (Figure 3-7). In the T157 transgenic cortex, there is a dynamic pattern of spatial-temporal expression of the RbP-LacZ reporter, the activity of which can be seen as early as E10.5 with an increase in activity as development progresses. T157 cortices from E13.5 to E16.5 were stained for RbP-LacZ activity (Figure 2-9). There is an increase in the intensity of the RbP-LacZ activity with developmental age. At E13.5, RbP-LacZ activity is restricted to the sub-pallial region, but by E14.5 a stripe of dark blue staining appears within the rostral-lateral cortex and grows in size and intensity by E16.5. Thus, Rb reporter expression correlates with increasing maturation of the mouse neocortices.

Since RbP-LacZ activity increased with developmental time, we postulated that high Rb expression might be associated with increased differentiation and maturation of neurons. Therefore, we Xgal stained micro-dissected adult coronal brain sections, where the majority of the cells are post-mitotic. RbP-LacZ activity is maintained within certain regions of the adult cortex (Figure 2-10). It is interesting that RbP-LacZ activity is seen both in the layers of the cerebral cortex, where mature neurons reside, as well as in the hippocampus and dentate gyrus, where adult neurogenesis occurs (Figure 2-10 C). These two regions are developed from the embryonic neocortex, while the thalamus is developed from the mesencephalon (midbrain) and the pons from the metencephalon (brain stem). The Xgal staining pattern in the adult is not pan-neuronal, and there is stronger RbP-LacZ activity in the rostral neocortex. This staining pattern demonstrates that high Rb levels are not a general marker of differentiated neurons but that Rb expression may play specific roles in specific cell types within the adult cortex. The localization of the RbP-LacZ activity to regions of embryonic and adult neurogenesis makes the cortex an interesting model for studying how loss of Rb family members affects the activity of the RbP-LacZ reporter.

**RbP-LacZ activity in the p130-deficient cortex**

Loss of p130 or p107 did not have a consistent effect on RbP-LacZ expression when analyzed by whole mount embryo analysis. To look more specifically at the domains expressing RbP-LacZ within the cortex, Xgal staining was performed on isolated cortex at E14.5. T157;p130+/- mice were crossed to p130+/- mice for timed pregnancies and embryonic cortices were micro-dissected at E14.5, followed by staining for LacZ activity. When comparing T157;p130+/- cortex to T157;p130/- cortex, there is either no
Figure 2-9: *RbP-LacZ* activity within the cortex increases with developmental age. Micro-dissected cortices from *T157* embryos at different time points are shown after Xgal staining. Olfactory lobes are oriented to the left. While only weak staining is seen within the cortex at E13.5, a blue region begins to appear in the pallium at E14.5, which increases in intensity and size as development progresses. (R= rostral, C=caudal) Legend bar= 1mm
Figure 2-10: *RbP-LacZ* activity is seen within different regions of the adult cortex.

Three 2mm thick coronal brain sections from a 2 month *T157* mouse were stained with Xgal for 6 hours. *RbP-LacZ* activity is seen both within the cerebral cortex, which contains a variety of types of mature neurons, and within the hippocampus, one of the sites of adult neurogenesis. *RbP-LacZ* shows stronger activity within more rostral regions (B) than in more caudal regions (D) of the adult brain. Derivatives of the midbrain (thalamus and hypothalamus) and brain stem (pons) show moderate staining. Bar = 1mm
change or a slight decrease in \(RbP\)-LacZ activity with \(p130\) deficiency (Figure 2-11). Due to the large variation in \(RbP\)-LacZ activity in within each genotype, it is difficult to discern if the decrease in \(RbP\)-LacZ activity is due to loss of \(p130\) or due to another variable like genetic background. Yet, loss of \(p130\) in the embryonic cortex or in the whole embryo does not show an increase in \(Rb\)-LacZ activity, and therefore \(p130\) is unlikely acting as a repressor of the \(Rb\) promoter during embryonic development. Additionally, two litters were dissected from crosses between \(T157; p107^{+/}\) and \(p107^{-/-}\) mice, but no \(T157; p107^{-/-}\) embryos were obtained.

\(RbP\)-LacZ activity with combined loss of \(p107\) and \(p130\) in the E14.5 cortex

Contrary to loss of \(Rb\), dual loss of \(p107\) and \(p130\) led to a decrease in \(RbP\)-LacZ activity within the developing nervous system in whole embryos. To better visualize the cortical regions displaying this decrease in \(RbP\)-LacZ activity, \(T157; p107^{+-}; p130^{-/-}\) mice were crossed to \(p107^{+/--}\) mice for timed pregnancies and embryonic cortices were analyzed for LacZ activity at E14.5. Overall, in 4 of 5 \(p107^{-/-}; p130^{-/-}\) cortices, there was a decrease in the activity of \(RbP\)-LacZ in the developing cortex relative to that seen in the \(T157; p107^{+/-}; p130^{-/-}\) cortex (Figure 2-12 B). This decrease was also seen in 2 of 3 \(T157; p107^{-/-}; p130^{-/-}\) cortices stained at E13.5 (not shown). The decrease in \(RbP\)-LacZ activity within the \(T157; p107^{-/-}; p130^{-/-}\) cortex is especially apparent within a streak of Xgal+ cells in the rostral pallium, which is much more prominent in the \(T157; p107^{+/-}; p130^{-/-}\) cortex (Figure 2-12 A).

**RbP-LacZ activity in the conditionally Rb-deficient cortex at E14.5**

Conditional loss of \(Rb\) within the nervous system led to an increase in \(RbP\)-LacZ activity within the nervous system by whole embryo analysis. To visualize this increase in the isolated cortex, \(NestinCre; RbLoxP/+\) mice were crossed to \(T157; RbLoxP/LoxP\) mice for timed pregnancies and embryonic cortices were micro-dissected and stained for LacZ activity at E14.5. Neuronal specific loss of \(Rb\) led to an increase in \(RbP\)-LacZ activity within the cortex (Figure 2-13). The increase in \(RbP\)-LacZ activity in embryonic cortex is similar to that seen by whole mount staining of \(Rb\) deficient embryos, in that there is a more intense and larger Xgal positive region within the cortex, but Xgal staining of the isolated cortex reveals that the increase in activity is regionally localized to an Xgal+ region within the rostral cortex. While the activity of \(RbP\)-LacZ within the caudal region does not appear to change with loss of \(Rb\), there is a dramatic increase in staining within the rostral pallium. Therefore, the increase in \(RbP\)-LacZ...
Figure 2-11: Moderate decrease in RbP-LacZ activity in the T157;p130-/- cortex at E14.5. Micro-dissected cortices from a T157;p130+/+ x p130-/- cross were stained with Xgal for 6 hours. Each hemisected cortex (A – F) represents a separate embryo from one litter. The T157;p130-/- cortices (D,E,F) show a decrease in RbP-LacZ activity compared to T157;p130+/+ cortices, although there is large variation within each genotype (columns). No wild type (T157;p130+/+) cortex was obtained in this litter.
Figure 2-12: Decreased RbP-LacZ activity in the E14.5 cortex with loss of p107 and p130. Micro-dissected cortices were obtained from 4 different litters from T157;p107+/−;p130−/− x p107+/−;p130−/− crosses and analyzed by Xgal staining. In panel A, each hemisected cortex represents one embryo from the same litter. Both T157;p107−/−;p130−/− cortices show reduced RbP-LacZ activity. Panel B shows the number of litters and embryos analyzed for each genotype.
activity with loss of \textit{Rb} does not appear to be due to a global de-repression of the reporter, but rather is specific to certain regions of the developing cortex. This increase could be due to a direct de-repression of \textit{RbP-LacZ} activity within the individual neurons; supporting the idea that pRB is a repressor of its own promoter. Alternatively, since the increase in staining appears to be primarily within the developing pallium, it is possible that deregulation of the cell cycle or differentiation has led to an increase in the production of specific \textit{RbP-LacZ} expressing neurons within the cortex.

\textbf{Summary}

The deregulation of the \textit{RbP-LacZ} reporter in embryos and cortices lacking various \textit{Rb} family members indicates that \textit{Rb} family members do play a role in controlling \textit{Rb} expression in vivo. Whether this is due to direct action at the \textit{Rb} promoter or due to indirect effects of \textit{Rb} family loss will be explored in the next chapter. It does appear that \textit{Rb} acts as a repressor of the \textit{Rb} promoter in vivo, because conditional loss of \textit{Rb} in the nervous system consistently led to an increase in activity from the \textit{RbP-LacZ} transgene in the developing cortex. The decrease in \textit{RbP-LacZ} activity with dual loss of \textit{p107} and \textit{p130} was surprising, because \textit{p107} and \textit{p130} are known as repressors of E2F-responsive genes. However, the decrease in \textit{RbP-LacZ} activity within the cortex implicates \textit{p107} and \textit{p130} as important for activation of the \textit{Rb} promoter. While \textit{p107} and \textit{p130} may directly recruit activators to the \textit{Rb} promoter, there are many other ways that \textit{p107} and \textit{p130} could be important for \textit{Rb} promoter activation. Loss of \textit{p107} and \textit{p130} could lead to the up regulation of additional repressors, including \textit{E2f6}, which is also an E2F target (Lyons et al., 2006) that subsequently could repress the \textit{Rb} promoter.

It is interesting that deregulation of \textit{RbP-LacZ} activity, using the \textit{T157 RbP-LacZ} reporter, with loss of various \textit{Rb} family members is spatially restricted within the cortex. This implies that \textit{Rb} promoter regulation by \textit{Rb} family members may be limited to certain neuronal populations in the E14.5 embryo. Although we did not perform these experiments using multiple \textit{Rb} promoter reporter lines, since the cortex showed the most consistent staining between the multiple wild type reporter lines (Agromayor et al., 2006), we believe that it is not a reporter specific effect. It is possible that complete de-regulation of \textit{RbP-LacZ} activity would require loss of all three \textit{Rb} family members. Additionally, the presence or absence of additional co-factors, repressors, or activators of the \textit{Rb} promoter within the different regions of the cortex may modulate any effect \textit{Rb} family loss may have on \textit{Rb} expression.
Figure 2-13: Neuronal specific loss of Rb increases RbP-LacZ activity in the cortex at E14.5

Micro-dissected cortices obtained from a T157;RbLoxP/+ x Nestin-Cre;RbLoxP/LoxP cross were stained for RbP-LacZ activity with Xgal. Each cortex represents an individual embryo from the same dissection. Rb mutant cortices (C and D) show much stronger staining within the pallium when compared to wild type controls (A and B). The increase in staining is seen particularly within the rostral regions of the cortex. (R= rostral, C= caudal)
Chapter 3: Direct Versus Indirect Effects of Rb Family Inactivation on the Rb Promoter

Direct Effects of Rb Family Members on the Rb Promoter

- ChIP in wild type cortex
- E14.5 cortex
- Juvenile cortex
- ChIP in Rb family deficient cortex (Rb, p107, p130, and p107;p130)

Indirect Effects of Various Rb Family Member Deficiencies on Cortical Development

- Analysis of cortical size
- Proliferation in various Rb family mutants
- Apoptosis in various Rb family mutants
- Changes in gene expression in various Rb family mutants
  - Analysis of genes required for cortical development
  - Analysis of E2F targets

Summary

Since loss of Rb family members in vivo had opposing effects on the activity of the RbP-LacZ reporter, we questioned whether the changes in RbP-LacZ activity were due to direct or indirect effects on the Rb promoter from Rb family member deficiency. Therefore, to test for direct regulation of the Rb promoter, occupancy of the Rb promoter was analyzed by chromatin immunoprecipitation (ChIP) in wild type and Rb family deficient cortical extracts. Loss of Rb family members may lead to aberrations in neuronal development, including changes in proliferation and cell death. Additionally, expression of many E2F responsive genes may be deregulated. Therefore, to look for indirect effects of Rb family loss within the embryonic cortex, changes in cortical size, proliferation (BrdU), apoptosis (TUNEL) and gene expression (RT-PCR) were analyzed.

Direct Effects of Rb Family Members on the Rb Promoter

Recent ChIP experiments in fibroblasts have demonstrated that while p130 occupies promoters in quiescence and G1, p107 can be found on promoters primarily during early G1 (Balciunaite et al., 2005). There is overlap between the promoters that p107 and p130 occupy, in agreement with the idea that these proteins can functionally compensate for each other. Interestingly, while loss of Rb alone has been shown to deregulate many genes (Markey et al., 2007), including known E2F targets, pRB has been conspicuously absent from promoters when analyzed by ChIP. One study has shown that pRB can be
Figure 3-1: Conserved E2F site of the Rb promoter lies within a GC rich region upstream of exon 1. The E2F site of the Rb promoter lies 180bp upstream of the translational start site at exon 1. The upstream region of the Rb promoter is highly GC rich (70.4%) (red). Primers which directly flanked the cassette failed to amplify a product using PCR analysis, while neighboring primers were able to consistently amplify a product despite the GC content. All ChIP experiments in embryonic cortex were performed with primers RbChIP10/RbChIP8.
found at E2F target promoters with E2F1 during S-phase (Wells et al., 2003) in Raji cells, a lymphoma cell line, while another group has shown pRB can be found with CBFA1 on promoters involved in osteogenesis in the osteoblast cell line, MC3T3-E1 (Thomas et al., 2001). Additionally, selective binding of pRB, and not p107 or p130, occurs at E2F targets involved primarily in DNA replication during senescence in human IMR90 cells (Chicas et al., 2010). Whether pRB can be found at promoters appears to be dependent on what cell line is being used, which promoters are being studied, and whether the cells are actively proliferating. Therefore, to test whether pRB is a direct repressor of the Rb promoter, it was important to perform ChIP using cortex because this is where RbP-LacZ activity is seen in vivo.

**ChIP analysis of the Rb promoter within wild type embryonic and juvenile cortex**

**ChIP analysis of the Rb promoter in the E14.5 embryonic cortex**

To determine if Rb family members are found at the Rb promoter in the E14.5 cortex, we performed ChIP on micro-dissected cortices from E14.5 C57BL/6 embryos. Due to the high G/C content of the Rb promoter, the only reliable primers for amplification were ~210bp upstream of the crucial E2F site in the regulatory cassette (Figure 3-1), but this was within the range of DNA fragments resulting from sonication (250-1000bp). Indeed, all three Rb family members occupy the Rb promoter in the E14.5 cortex (Figure 3-2). The strength of the pRB signal did vary between experiments, but on a C57BL/6 background, occupancy by pRB was seen in 3 of 4 separate ChIP experiments and with two different antibodies. p107 and p130 were found at the Rb promoter in 2 of 2 separate ChIP experiments, although the signal was weaker than that for pRB. To contrast this with another promoter, the p107 promoter, which contains tandem E2F sites (L. Zhu et al., 1995) and is a well-recognized E2F target in fibroblasts, was also analyzed for Rb family occupancy. Surprisingly, we never found occupancy by Rb family members at the p107 promoter in the E14.5 cortex (Figure 3-2). Dihydrorofolate reductase (Dhfr) is a known E2F regulated gene, which contains two overlapping E2F sites, and has been shown to be regulated by E2F family members in culture systems (reviewed in Abali et al., 2008). In E14.5 cortex, we consistently found binding of Rb family members, p107 and p130, at the Dhfr promoter, indicating that there is differential regulation of E2F targets by Rb family members in the developing cortex. An attempt to quantify Rb family occupancy at the Rb promoter was performed using RT-PCR, but difficulties in amplification using the same primers precluded any conclusions (See Appendix).
Figure 3-2: *RB* family members occupy the *Rb* promoter in E14.5 wild type cortex.

ChIP was performed on E14.5 embryonic cortex using antibodies to different pocket proteins and histone markers. All three pocket proteins can be found at the *Rb* promoter. The *Dhfr* promoter, which contains known E2F binding sites, shows a different pattern of pocket protein binding than at the *Rb* promoter. In contrast, the *p107* promoter, which contains two tandem E2F sites, shows no pocket protein binding in E14.5 cortex. Amplification of a region within *Rb* intron 3 was performed to show that the binding is specific to the *Rb* promoter.
We also monitored histone marks at the Rb promoter, and there was a strong enrichment for trimethylated lysine at position 4 in histone 3, denoted TriMetH3K4, and acetylation of histone H3 (denoted as AcH3), indicating an open chromatin structure and an active gene (Santos-Rosa et al., 2002). We also found enrichment for trimethylated lysine at position 9 in histone 3 (and potentially lysine 27 due to cross reaction of the antibody), denoted as TriMetH3K9/27, which is a histone mark of condensed chromatin and gene silencing (Nakayama et al., 2001). An active Rb promoter, as represented by RbP-LacZ activity, was restricted to certain regions of the cortex, and so it is not surprising to see both active and repressive marks at the Rb promoter in the mixed population of the cortex. The presence of pRB family members at the Rb promoter in E14.5 cortex strongly suggests that Rb family members are having a direct effect on Rb transcription. Yet, while we would predict from this that loss of any of the Rb family members would lead to an increase in Rb expression, we see that loss of Rb and dual loss of p107 and p130 have opposing effects on RbP-LacZ activity in the cortex. Therefore, there must be more than one mechanism that explains the downstream effects of Rb family loss on RbP-LacZ activity.

ChIP analysis of the Rb promoter in the juvenile cortex

Since RbP-LacZ activity is still maintained within the adult cortex, we performed ChIP on chromatin derived from 5-week C57BL/6 mouse cortex using antibodies against pRB and E2F family members. We found that both E2F and pRB family members are still found at the Rb promoter in the juvenile cortex (Figure 3-3). Immunoprecipitation using an antibody specific for TriMetH3K4 showed strong enrichment at the Rb promoter, indicating that this promoter is indeed active in the juvenile cortex. The TriMetH3K4 signal was absent from the Cdc2 promoter, which is not expected to be active within most cells of the juvenile cortex (Okano et al., 1993). Diverse E2F family members can be found at the Rb promoter: E2F1 and E2F2, which form complexes primarily with pRB, E2F4, which can be found in complex with all three pocket proteins, and E2F6, which lacks the pRB binding domain. We attempted to find E2F-2, E2F-3, and E2F-5 at the Rb promoter, but we did not find occupancy by these E2F family members in the juvenile cortex. The predominant Rb family members found at the Rb promoter in the juvenile cortex are pRB and p130, with p107 rarely found in the juvenile cortex. Unlike in the embryonic cortex, we found that the RB-1 antibody did not appear to perform as well as RB-2 in ChIP at the Rb
Figure 3-3: E2F and pRB family members occupy the Rb Promoter in the juvenile cortex. Two separate ChIP experiments were performed on chromatin from 5-week old mouse cortex. E2F1, E2F2, E2F4, and E2F6 all can be found at the Rb promoter in the juvenile cortex in addition to p130 and pRB. Binding of p107 was not found at the Rb promoter within the juvenile cortex. RB-1 and RB-2 refer to two different polyclonal antibodies specific to pRB. The Cdc2 promoter, which should not be expressed strongly within the juvenile cortex, shows a different pattern of binding for E2F and RB family members, as well as little trimethylation of Histone H3 at lysine 4 (TriMet-H3K4).
promoter, potentially due to the different procedures required for cross-linking chromatin from juvenile cortex compared to embryonic cortex. Therefore, regulation of the Rb promoter by pRB and E2F family members occurs in the juvenile cortex, where we see strong RbP-LacZ activity.

**Analysis of the Rb promoter by ChIP in the various Rb family deficient cortices at E14.5.**

Changes at the Rb promoter with loss of Rb family members could explain how RbP-LacZ activity changes within the cortex. Some E2F targets have been shown to be occupied by multiple Rb family members. For example, both p107 and p130 have been found at the E2f1 promoter in T98G glioblastoma cell line, and therefore loss of only one Rb family member may not affect gene expression because another Rb family member may take its place at the promoter (Takahashi et al., 2000). Additionally, novel complexes between activator E2Fs and p107 and p130 have been found in Rb deficient MEFs, indicating that loss of Rb family members may lead to compensation or shuffling of repressive complexes at promoters (E.Y. Lee et al., 2002).

**Analysis of the Rb promoter in Rb-deficient cortex**

To test if loss of Rb leads to a shuffling of pocket protein binding at the Rb promoter, we performed comparative ChIP analysis on cortical chromatin isolated from wild type RbLoxP/+ and NestinCre;RbLoxP/LoxP embryos at E14.5. Clearly, there is loss of pRB binding in the NestinCre;RbLoxP/LoxP cortex, indicating that the RB-1 antibody is indeed specifically detecting pRB (Figure 3-4). While we did not observe p107 binding in this experiment, p130 binding is seen both in the RbLoxP/+ and NestinCre;RbLoxP/LoxP cortex (p107 found in one of two separate experiments; p130 found in two of two separate experiments). Both E2F1 and E2F4 can be found at the Rb promoter in wild type and Rb deficient cortex; however, more E2F4 is bound in the NestinCre;RbLoxP/LoxP cortex compared to that bound in RbLoxP/+ . Loss of pRB binding at the Rb promoter could explain part of the increase in RbP-LacZ activity seen in the cortex with loss of Rb. Yet, the continual presence of p130 and E2F4, known repressors of gene activity, at the Rb promoter in Rb deficient cortex suggests that loss of pRB binding alone may not be the only driving factor leading to the increase in RbP-LacZ activity with loss of Rb.
Figure 3-4: Moderate changes at the Rb Promoter with loss of Rb in E14.5 cortex.
ChIP was performed on chromatin obtained from E14.5 embryonic cortices from wild type and Nestin-Cre;RbloxP/loxP littermate. The presence of pRB at the Rb promoter is lost in Rb deficient cortex and an increase in E2F4 suggests reshuffling of complexes at the Rb promoter.
Analysis of the Rb promoter in p107-deficient cortex

Loss of p107 or p130 alone did not lead to strong changes in RbP-LacZ activity within the embryo, although both pocket proteins can be found at the Rb promoter by ChIP (Figure 3-3). Both p107 and p130 regulate an overlapping set of genes (Balciunaite et al., 2005) and therefore loss of only p107 or p130 might not be expected to cause large changes in promoter occupancy. Although little p107 binding to the Rb promoter was detected in wild type and p107/- cortex, inactivation of p107 induced an increase in p130 binding at the Rb promoter in the mutant cortex (Figure 3-5). A similar increase in p130 binding occurs at the Dhfr promoter, indicating that there is some shuffling of pocket protein members with loss of p107 alone. pRB is found at the Rb promoter in both p107+/+ and p107/- cortex. We found no loss of histone H3 acetylation or trimethylation at histone H3 lysine 4.

Analysis of the Rb promoter in p130 and p107;p130-deficient cortex

Analysis of pocket protein binding at the Rb promoter in p130 deficient cortex was hindered by the absence of Rb family occupancy within the wild type cortex. What we can see is a moderate increase in the occupancy of E2F4 and a decrease in trimethylation at histone H3 lysine 4, in the absence of p130 (Figure 3-6A). This is an indication that the Rb promoter may be less active with p130 deficiency. Binding of p107 to the Rb and Dhfr promoters increased with p130 deficiency, although this was minimal at the Rb promoter, indicating that there is some shuffling in E2F family members at promoters with p130 deficiency. E2F4 binding decreased to the Cdc2 promoter with p130 deficiency, but this was not accompanied by an increased TrimethylH3K4 mark, which would signal an active promoter.

We performed ChIP on chromatin from p107+/+;p130-/- and p107-/-;p130-/- cortex, and found moderate changes at the Rb promoter (Figure 3-6 B). While we see p107 binding at the Rb promoter in the p107+/+;p130-/- cortex, we no longer found binding in p107-/-;p130-/- cortex (as compared to IgG control). While it appears that pRB binding is increased in p107-/-;p130-/- cortex, the high IgG background does not allow us to make this conclusion. Therefore, it does not appear that changes in pocket protein occupancy at the Rb promoter can alone explain the decrease seen in RbP-LacZ activity within the embryo. It is interesting that there is little acetylated histone H3 at the Rb promoter in both the p107+/+;p130-/- and p107-/-;p130-/- cortex, since strong acetylated histone H3 was consistently found in wild type and Rb deficient cortex. This is an indication of a less open chromatin structure at the Rb
Figure 3-5: Moderate changes at the Rb Promoter with loss of p107 in E14.5 cortex.

ChIP was performed on wild type and p107/- cortex from E14.5 littermates. There is a moderate increase in p130 occupancy in p107 deficient cortex. There appears to be no change in pRB occupancy.
promoter with loss of p130 or dual loss of p107 and p130, which could be contributing to the decrease in Rb expression in the p107/-;p130/- cortex. The Dhfr promoter, which shows no great change in trimethylation at H3K4, displayed decreased acetylation of histone H3 in the p107/-;p130/- cortex. So while there is little shuffling in pocket protein occupancy at the Rb promoter with dual loss of p107 and p130, loss of active histone marks at the Rb promoter could explain the decrease in RbP-LacZ activity in the p107/-;p130/- cortex. Since there is also a loss of active marks at the Dhfr promoter, this could be a more general phenomenon occurring at E2F targets in the p107/-;p130/- cortex.

While Rb family members can be found at the Rb promoter in the embryonic cortex, the small changes in pocket protein occupancy in Rb family deficient cortices appear insufficient to completely explain the differences seen in RbP-LacZ activity within the cortex. The maintenance of both E2F1 (an activator E2F) and E2F4 (a repressor E2F) at the Rb promoter in Rb deficient cortex demonstrates that the presence of both active and repressive E2F complexes remain at the Rb promoter with Rb loss, with potentially an increase in E2F4 repressive complexes. It would be interesting to know whether the presence of other E2F family members at the Rb promoter in the embryonic cortex changes with loss of Rb family members. Specifically, there may be changes in the occupancy of E2F6, E2F7, and E2F8, since they function as repressors in a pocket protein independent manner. While not conclusive, loss of p107 and p130 appeared to lead to a decrease in active histone marks (AcH3 and TriMetH3K4) at a variety of promoters, including the Rb promoter. Most Rb family interactions are known to be with cofactors that lead to the silencing of gene expression. Loss of p107 and p130 may lead directly to changes in recruitment of a histone modifying enzymes at E2F regulated promoters, including the Rb promoter. It would be important to know if loss of p107 and p130 directly affects Rb expression in the cortex. A more intensive study of histone marks, as well as the presence of histone modifying enzymes, in p107/-;p130/- cortex would reveal if loss of p107 and p130 leads to epigenetic changes at the Rb promoter.

**Evaluating Indirect Effects of Rb Family Member Loss on Rb Promoter Activity**

**Changes in cortical size with loss of Rb family members**

The best recognized role of Rb family members is in regulation of proliferation and cell cycle control. Therefore, loss of Rb, p107, or p130 could affect the number of cells that are produced in the cortex, leading to smaller or bigger brains. While analyzing RbP-LacZ activity in embryos, we observed
Figure 3-6: Small changes at Rb promoter in p130-/- and p107-/-;p130-/- cortex at E14.5. Panel (A) shows a ChIP experiment performed on wild type and p130-/- cortices from E14.5 litter mates. There is a drop in trimethylation at Histone H3K4 at the Cdc2 and Rb promoters with p130 deficiency. Panel (B) shows a ChIP experiment performed on cortex from p107+/+;p130-/- and p107-/-;p130-/- litter mates. A decrease in the presence of acetylated Histone H3 (AcH3) was seen at the Cdc2 and Dhfr promoter, as well as a decrease in trimethylation at Histone H3 at the Cdc2 promoter. Changes in RB family member occupancy was inconclusive due to weak binding seen within these experiments.
that NestinCre;RbLoxP/LoxP embryos appeared to have larger brains, as has been reported by MacPherson et al. (2003). When E14.5 cortices were micro-dissected and cortical cross-sectional area was calculated, there was a modest but consistent increase in the size of the cortex with loss of Rb in the nervous system (Table 3-1 A). We did notice that cortical area size varied depending on the direction of the matings, but the increase in cortical cross-sectional area with Rb loss was apparent whether Cre recombinase was inherited from the father or mother. An increase in cross-sectional cortical area size is consistent with a published telencephalic knockout of Rb, which showed a 30% increase in cortical area at E16.5 using a Foxg1-Cre conditional Rb mutant (Ferguson et al., 2002). Therefore, the increase seen in RbP-LacZ activity with loss of Rb may be due in part to an increase in neurogenesis, which indirectly leads to an increase in the number of RbP-LacZ expressing neurons within the cortex. While loss of p107 or p130 alone is not known to have large effects on the cell cycle, loss of both p107 and p130 has been shown to cause changes in cell cycle dynamics in mouse embryonic fibroblasts (MEFs) (Classon et al., 2000b). Analogous changes in cell cycle dynamics in the CNS could also lead to changes in brain sizes. To test if loss of both p107 and p130 changed cortical size, embryonic cortices from both p130+/- and p107+/-;p130-/- crosses were micro-dissected and cross-sectional cortical area was measured at E14.5. However, there was no significant change in cortical area with either loss of p130 alone or dual loss of p107 and p130 (Table 3-1 B and C). Therefore, the decrease in RbP-LacZ activity in p107-/-;p130-/- cortex is not due to differences in brain size. Additionally, although loss of p107 and p130 are known to lead to deregulation of the cell cycle and to an increase in proliferation similar to loss of Rb, dual loss of p107 and p130 does not lead to a similar increase in embryonic cortical size.

Changes in proliferation in various Rb family deficient cortices at E15

Effect of p107 or p130 loss on proliferation in the E15 cortex

The embryonic cortex at E15 is composed of multiple cell types and has a distinct laminar structure that is formed in an “inside-out” method, where the first-born neurons make up the outer most regions of the cerebral cortex (Figure 3-7). New cells are born within the ventricular and sub-ventricular zones consisting of progenitors at various states of commitment to different neuronal fates. As neurons become post-mitotic, they migrate to their proper layer within the cortex and become further committed to specific neuronal cell fates (reviewed in Dehay and Kennedy, 2007). To test the effect of loss of Rb family
Table 3-1: Changes in cortical area with loss of Rb family members at E14.5

The cross-sectional area in the sagittal plane of cortical hemispheres was measured using ImageJ using an internal scale. The change in area between genotypes is expressed as a ratio of the average area of the Rb family deficient cortex (mutant) over the average area of wild type littermates (WT). The Rb deficient cortex (A) showed a small but significant increase in cortical area size (Students paired t-test $p=0.004$). There was no significant change in cortical area with loss of $p130$ (B) or dual loss of $p107$ and $p130$ (C).

<table>
<thead>
<tr>
<th>Cre Source</th>
<th>$Rb\text{LoxP/LoxP}$ or $Rb\text{LoxP/+}$ (mm$^2$)</th>
<th>$\text{Nes-Cre;RbLoxP/LoxP}$ or $Rb\text{LoxP/X}$ (mm$^2$)</th>
<th>Ratio (Mutant/WT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paternal</td>
<td>6.1±0.1 (n=5)</td>
<td>6.7±0.3 (n=5)</td>
<td>1.10</td>
</tr>
<tr>
<td>Maternal</td>
<td>7.4±0.2 (n=4)</td>
<td>8.0±0.4 (n=6)</td>
<td>1.08</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average Area</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$p130$ +/- or $p130$ +/-(mm$^2$)</td>
<td>$p130$ -/- (mm$^2$)</td>
<td>Ratio (Mutant/WT)</td>
</tr>
<tr>
<td>6.2±0.3 (n=5)</td>
<td>5.9±0.3 (n=4)</td>
<td>0.95</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Average Area</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>$p107$ +/-; $p130$ -/- (mm$^2$)</td>
<td>$p107$ -/-; $p130$ -/- (mm$^2$)</td>
<td>Ratio (Mutant/WT)</td>
</tr>
<tr>
<td>6.2±0.2 (n=10)</td>
<td>6.2±0.3 (n=7)</td>
<td>0.998</td>
</tr>
</tbody>
</table>
Figure 3-7: Development of the laminated structure of the neocortex is a dynamic process. A schematic of a coronal section of an E14 brain is shown (A). Interneurons develop within the ventral telencephalon and migrate tangentially into the neocortex. Projection neurons develop from progenitors within the dorsal telencephalon and migrate radially into the neocortex. A magnified view of the region inside the rectangle drawn in (A) is shown in (B). The neocortex develops by an outside-in mechanism, with the earliest born neurons positioned within the outer cortex. Neuronal progenitors reside within the ventricular (VZ) and subventricular (SVZ) zones, where they divide, and then migrate into the upper layers of the cortex to establish the proper neuronal connections. VZ = ventricular zone; PP = preplate; SVZ = subventricular zone; SP = subplate; CP = cortical plate; MZ = marginal zone; FL = Fibre Layer.

Adapted with permission from Dehay and Kennedy, Nature Reviews Neuroscience (June 2007)
members on proliferation in the cortex, we analyzed BrdU incorporation at E15 by histological analysis on the different mutant backgrounds. Heterozygous p107 or p130 mice were set up for timed pregnancies and pregnant females were injected with BrdU one hour before sacrifice on Day E15. Serial sections were processed for BrdU incorporation from wild type and mutant embryos, and comparisons were made between genotypes by gauging the strength and localization of the BrdU signal within the ventricular and subventricular zones of the neocortex in multiple sections. Sections between embryos were matched using the size of the ventricle. With loss of p107 there was a modest decrease in BrdU uptake within the ventricular zone (n=2 embryos per genotype) (Figure 3-8 A vs. B). This was unexpected based on the recognized role of p107 as a suppressor of proliferation and given that previously, another group has reported that loss of p107 using a different mouse model led to an increase in BrdU incorporation within the ventricular zone (Vanderluit et al, 2007). Again, contrary to its known role as a suppressor of proliferation, we also found a modest decrease in BrdU uptake within the ventricular and subventricular zones of the p130-/- cortex (n=3 embryos per genotype) (Figure 3-8 C vs. D). Thus, loss of p107 or p130 alone both lead to decreases in proliferation in the E15 cortex.

Effect of dual loss of p107 and p130 on proliferation in the E15 cortex

To determine the effect of combined loss of p107 and p130, we crossed p107+/-;p130-/- mice for timed pregnancies, and embryos were collected as previously described. We found that combined loss of p107 and p130 led to a modest increase in BrdU incorporation within the ventricular zone as assessed by the strength of the BrdU signal (n=2 embryos per genotype) (Figure 3-9), agreeing with previous data that it takes both loss of p107 and p130 to cause significant deregulation of proliferation (Hurford et al., 1997; Classon et al., 2000). Yet, this increase in proliferation within the ventricular zone does not seem to be much higher than wild type levels and there is no ectopic proliferation within the cortex. Therefore, while the combined loss of p107 and p130 leads to more proliferation, it is a very minimal effect. We also observed that there was a change in the positional staining of the BrdU+ cells with loss of p130 and dual loss of p107 and p130 (Figures 3-8 and 3-9). Since the width of the proliferative zones (VZ and SVZ) are dynamic as one progresses medially (towards the midline) within the cortex, it was difficult to determine whether this change in position was due to differences in genotype or variation in sectioning between embryos. Loss of p107 or p130 resulted in a decrease in proliferation within the cortex, but did not have a
Figure 3-8: Loss of *p107* or *p130* leads to a moderate decrease in proliferation in the E15 cortex. Incorporation of BrdU in E15 cortices was analyzed in *p107* and *p130* deficient embryos. Loss of *p107* (B) showed a decrease in BrdU incorporation within the subventricular zone (SVZ) and ventricular zone (VZ) of the developing cortex as compared to wild type litter mate (A). The *p130* deficient cortex (D) showed a similar decrease in BrdU incorporation as compared to wild type litter mate (C). The decrease was seen in at least 2 separate experiments for each genotype. SP=subplate.
Figure 3-9: Dual loss of $p107$ and $p130$ leads to a moderate increase in proliferation in the E15 cortex. BrdU incorporation was analyzed in E15 cortices of $p107^{-/-};p130^{-/-}$ (B) embryos and compared to litter mate $p107^{+/+};p130^{-/-}$ (A) embryos. A small but consistent increase in was seen in BrdU incorporation in two separate experiments.
Figure 3-10: Ectopic proliferation with conditional loss of Rb in the E15 cortex.

Increased BrdU incorporation was seen within the developing cortex of a conditional Rb deficient embryo. The wild type litter mate RbLoxP/LoxP (A) shows BrdU incorporation within the normal proliferating zones of the cortex, the ventricular zone (VZ) and the subventricular zone (SVZ). The NestinCre;RbLoxP/LoxP cortex (B) shows BrdU incorporation in the VZ, SVZ, as well as in normally non-proliferative zones including the sub-plate (SP). Ectopic proliferation was seen in 2 separate experiments.
large effect on RbP-LacZ activity, while dual loss of p107 and p130 has a minimal effect on proliferation within the cortex but led to a decrease in RbP-LacZ activity. For that reason, it does not appear that a change in proliferation is a major contributor to the diminished activity of the RbP-LacZ reporter in the p107-/-;p130-/- cortex.

Effect of conditional loss of Rb on proliferation in the E15 cortex

Constitutive loss of Rb in the germline results in ectopic proliferation outside of the ventricular and subventricular zones of the cortex (E.Y. Lee et al., 1992; Clarke et al., 1992; Jacks et al., 1992). To test if this occurs with neuronal specific loss of Rb, we analyzed BrdU incorporation in NestinCre;RbLoxP/LoxP embryos at E15, as previously described. Indeed, we found that BrdU incorporation is seen in many layers of the developing cerebral cortex of the NestinCre;RbLoxP/LoxP cortex (Figure 3-10 A), which we did not find within wild type embryos (Figure 3-10 B) (n=3 embryos per genotype). This data agrees with previously reported increase in ectopic S phase entry using an alternate NestinCre system to inactivate Rb (MacPherson et al., 2003). An increase in proliferation within the cortex, with the concurrent increase in cortical size, is an indicator that there is an increase in neurogenesis with loss of Rb. Therefore, it is possible that the enlarged cortex may contain more neurons expressing the RbP-LacZ transgene. This possibility will be discussed further in the following chapter.

Changes in apoptosis in various Rb family deficient cortices at E15

Effects of loss of p107 or p130 on cell death in the E15 cortex

Apoptosis is an essential part of the proper formation of the cerebral cortex, and it is particularly important for the proper establishment of synaptic connections between mature neurons (Burek and Oppenheim,. 1996). Using the TUNEL assay on histological sections of the wild type embryonic cortex at E15, we found that the majority of apoptotic cells are within the marginal zone, cortical plate, and subplate, the layers in which mature neurons are found (Figure 3-11). To test if loss of Rb family members affects apoptosis within the cortex, we performed TUNEL assays using serial sections from the same E15 mutant embryo sets analyzed previously for BrdU incorporation. The amount of cell death was determined by intensity and location of TUNEL positive cells. In p107-deficient embryos, there was a clear decrease in the amount of TUNEL positive cells (Figure 3-11), signifying a decrease of apoptosis within the marginal zone and cortical plate (n=2 embryos per genotype). In p130-deficient embryos, there
Figure 3-11: Decreased cell death with loss of p107 in the developing cortex at E15.

Analysis of cell death was performed using the TUNEL assay on sections from E15 p107+/+ and p107-/- embryos. There was a consistent decrease in apoptotic cells with loss of p107 (D) within the marginal zone and cortical plate relative to the p107+/+ littermate (C) (n=2). Panels in which Terminal deoxynucleotidyl transferase (TdT) enzyme was not added (A and B) are shown to delineate cortical layers. MZ= marginal zone, CP= cortical plate, SP= subplate.
Figure 3-12: Decreased cell death with loss of p130 in developing cortex at E15.

Analysis of cell death was performed using the TUNEL assay on sections from E15 p130+/+ and p130/- embryos. There was a consistent decrease in apoptotic cells with loss of p130 (D) within the marginal zone, cortical plate, and the subplate of the developing cortex relative to a wildtype litter mate (C). Adjacent sections in which no enzyme (-TdT) is added are shown in panels (A) and (B) to delineate cortical layers. (n=2) MZ= marginal zone, CP= cortical plate, SP= subplate
was a dramatic decrease in TUNEL positive cells (Figure 3-12), particularly within the cortical plate and subplate (n=2 embryos per genotype).

**Effects of dual loss of p107 and p130 on cell death in the E15 cortex**

Loss of p107 and p130 led to a decrease in RbP-LacZ expression (Figure 2-3). One possible explanation for this effect could be that an increase in cell death led to a loss of RbP-LacZ expressing neurons. To determine if dual loss of p107 and p130 increased cell death within the cortex, we used TUNEL analysis to compare p107+/+; p130-/- and p107-/-; p130-/- embryos at E15 (n=2 embryos per genotype). We found that loss of both p107 and p130 led to a further decrease in TUNEL-positive cells within the cortical plate and subplate of the cortex (Figure 3-13). Therefore, loss of p107, p130, or both p107 and p130 leads to a pronounced decrease in apoptosis within the developing cortex at E15. Therefore, the decrease in RbP-LacZ activity within the p107-/-;p130-/- embryonic cortex is not due to a loss of RbP-LacZ expressing cells through cell death. The decrease in apoptosis does indicate that although p107-/-;p130-/- cortices appear grossly normal, loss of p107 and p130 is altering the dynamics of neuronal development. The large loss of cell death is especially surprising with loss of p107 or p130 alone since these mice live normal lifespans, although behavioral changes have not been assessed.

**Effect of conditional loss of Rb on cell death in the E15 cortex**

While an increase in proliferation could lead to more RbP-LacZ positive cells with the conditional loss of Rb, a decrease in cell death could achieve the same result. To see if this is the case, we used the TUNEL assay to analyze NestinCre;RbLoxP/LoxP embryos at E15 to look for changes in cell death within the neocortex. We found that there was either no change or a very slight decrease in apoptosis with loss Rb within the developing cortex (Figure 3-14) (n=3 embryos per genotype). Therefore, changes in cell death are most likely not the cause of the increased RbP-LacZ expression with loss of Rb.

**Investigation of changes in gene expression with various Rb family member loss**

A possible mechanism by which loss of Rb family members affects proper neuronal development is through deregulation of genes required for neurogenesis or E2F target genes, indirectly impacting the activity of the RbP-LacZ reporter. To test for deregulation of E2F responsive promoters, quantitative RT-PCR was performed on mRNA from E12.5 brains and E14.5 micro-dissected cortices from embryos lacking various Rb family members. At E12.5, brains from constitutive Rb knockout embryos were
Figure 3-13: Decreased cell death with dual loss of p107 and p130 in developing cortex at E15. Analysis of cell death was performed using the TUNEL assay on sections from E15 p107+/+;p130-/- and p107-/-;p130-/- embryos. A further decrease in apoptotic activity was seen in the cortical plate and subplate in p107-/-;p130-/- cortex (D) relative to the p107+/+;p130-/- littermate (C). Sections were boiled longer in citrate compared to single knockouts to recover a TUNEL positive signal. Adjacent sections in which no enzyme (-TdT) is added are shown in panels (A) and (B) to delineate cortical layers. MZ= marginal zone, CP= cortical plate, SP= subplate.
Figure 3-14: No change in cell death with conditional loss of \( Rb \) in the E15 cortex.

Analysis of cell death was performed using the TUNEL assay on sections from E15 Nestin-Cre;\( Rb\text{-LoxP}/\text{LoxP} \) embryos, and compared to either \( Rb\text{-LoxP}/+ \) or \( Rb\text{-LoxP}/\text{LoxP} \) litter mates. Apoptotic cells were detected primarily in the marginal zone (MZ), cortical plate (CP), and subplate (SP) of the developing cortex. There was no consistent change in cell death with loss of \( Rb \) (C vs D) (n=3). Adjacent sections in which no enzyme (-TdT) is added are shown in panels (A) and (B) to delineate cortical layers.
analyzed, but at E14.5 the Nestin-Cre conditional Rb knockout was used, since this is the time point at which Rbp-LacZ activity was monitored. Changes in expression level were determined by comparing the copy number levels of the target gene from mutant tissue to copy number levels from wild type tissue and expressed as a ratio. Copy numbers were normalized to levels of actin from concomitant runs.

**Effects of Rb family member loss on regulators of neuronal development**

Fgf growth factors (ligands) and their receptors are important for the proper differentiation and development of the telencephalon (reviewed in Hebert and Fishell, 2008; Mason, 2007). Additionally, Fgf2 is a possible E2F target important in neuronal development (McClellan et al., 2009). Levels of mRNA for Fgf ligands and Fgfr receptors from E14.5 cortices were measured in the various Rb family deficiencies, and normalized to levels of actin (internal control) (Table 3-2). There was no significant deregulation of any Fgf family members or Fgfr receptors in any of the mutant backgrounds tested.

The Hes transcription factor family is an important set of regulators within the Notch signaling pathway that is important for the regulation of neuronal precursor proliferation and differentiation within the telencephalon (Kageyama et al., 2008). Specifically, Hes1 can be negatively regulated by p107 (Vanderluit et al., 2007). However, we found no deregulation of Hes1, Hes5, or Hes6 at the mRNA level in E14.5 cortex with loss of various Rb family members (Table 3-2). Therefore, there is no global deregulation of the FGF or Notch-Hes pathways with loss of Rb family members, suggesting that neuronal development is not completely compromised by loss of Rb family members.

**Rb family member expression in cortex lacking various Rb family members**

Both the Rb (Zacksenhaus et al., 1993) and p107 (L. Zhu et al., 1995) promoters contain critical E2F binding sites and both have been shown to be E2F responsive genes in various systems (Burkhart et al., 2010b; E.J. Smith et al., 1998). On the other hand, p130 is not a known E2F regulated gene. Using RT-PCR, we measured the levels of Rb family mRNA normalized to actin levels in the cortex (E14.5) or brain (E12.5) of embryos lacking various Rb family members (Table 3-3). In E12.5 brains, we found no deregulation of Rb family members with the inactivation of Rb family members at the mRNA level (Table 3-3 A). A similar result was found using the E14.5 cortex (Table 3-3 B). It was surprising that Rb levels did not change in the p107-/-;p130-/- deficient cortex since Rbp-LacZ activity clearly decreased in the doubly deficient cortex. However, because the whole cortex is used to generate mRNA, and Rbp-LacZ activity is
### Table 3-2: Minimal Fgf, Fgfr or Hes family deregulation in E14.5 mutant cortex using real time RT-PCR.

Changes in mRNA levels of Fgfs, Fgf receptors, and Hes transcription factors in various RB family deficient cortices is given as the ratio of the target copy number in the mutant cortex divided by the target copy number of the respective wild type cortex. Target copy numbers are normalized to internal levels of actin and are averaged from at least three separate cortices per genotype (n=3-6). There were no significant changes in expression of any of the tested Fgf, Fgfr, or Hes genes.
Table 3-3: Absence of deregulation of RB family members using real time RT-PCR.

Changes in mRNA levels of Rb family members in the various RB family deficient cortices is given as the ratio of the target copy number in the mutant cortex divided by the target copy number of the respective wild type cortex. Target copy numbers are normalized to internal levels of actin and are averaged from at least three separate cortices per genotype (n=3-6).

No changes in expression of Rb family members were seen either in E12.5 brain (A) or E14.5 cortex (B). Decreases in the levels of each Rb family member was seen in the respective mutant, confirming the efficiency of the knockout models. The Rb constitutive knockout was used at E12.5, before embryonic lethality, and the conditional Nestin-Cre;RbLoxP/LoxP cortex was analyzed at E14.5.
restricted to a subset of cortical cells, a decrease in \( Rb \) levels within this subset of cortical cells may be masked by analysis of the whole cortex.

*E2f family member expression in cortex lacking various \( Rb \) family members*

Many *E2f* genes themselves are E2F targets (*E2f1* - Hsaio et al., 1994; Johnson et al., 1994; Neuman et al., 1995; *E2f2* - Sears et al., 1997; *E2f3a* - Leone et al., 2000; *E2f6* - Lyons et al., 2006; *E2f7* - DeBruin et al., 2003a; and *E2f8* - Christensen et al., 2005) and deregulation of *E2f* family members could affect *RbP-LacZ* expression indirectly by changing the balance of E2F family members or expression of their targets within the developing brain. We found that the majority of *E2f* family members were not deregulated within the E12.5 brain (Table 3-4 A). Additionally, mRNA levels of DP family members, *Dp1* and *Dp2*, which are the dimerization partners for E2F1-6, did not change with loss of *Rb* family members in the E12.5 brain. There was an increase in the level of *E2f8*, which is an *E2f* responsive gene, with loss of *Rb* or dual loss of *p107* and *p130*. In E12.5 brains, levels of *E2f8* were very low as observed from high C(t) values, which indicated that *E2f8* levels were not detectable until the very last amplification cycles. So analysis at another time point was conducted (E14.5) to explore this result. Additionally, both loss of *Rb* and loss of *p107* and *p130* led to an increase in *E2f8* levels at E12.5, which does not explain the opposing effects these genotypes have on *RbP-LacZ* activity in the cortex.

To determine whether deregulation of *E2f* family members occurs at E14.5, micro-dissected cortices were analyzed for levels of *E2f1*, *E2f4*, and *E2F8* in the different \( Rb \) family deficiencies (Table 3-4 B). There is deregulation of *E2f* family members with loss of *Rb* family members in the E14.5 cortex that was not seen at the earlier time point. Notably, there was a significant doubling in the levels of *E2f1* (students t test \( \rho=0.0009 \)), a known E2F target gene, in the conditional *NestinCre;RbLoxP/LoxP* cortex. This result is consistent with the increase in proliferation seen in the *Rb* deficient cortex. Also noteworthy was a decrease in levels of *E2f4* with loss of *p107* (student’s t test \( \rho=0.0013 \)). *E2f4* is not known to be an *E2f* target gene and expression of *E2f4* is not cell cycle regulated, so this could be an indirect effect of *p107* loss. At E14.5, the level of *E2f8* could be more accurately measured, and there was no significantly different change in *E2f8* with loss of *Rb* or dual loss of *p107* and *p130*. There was also moderate increases in *E2f1* levels in *p130/-,* p107/-,* or *p107/-;p130/-* cortex. Loss of *Rb* family members in the cortex leads to deregulation of *E2f* transcription factors, although the changes seen are modest. While an
### Table 3-4: Deregulation of E2f family members in E14.5 cortex.

Changes in mRNA levels of E2f family members in the various Rb family deficient cortices are given as the ratio of the target copy number in the mutant cortex divided by the target copy number of the respective wild type cortex. Target copy numbers are normalized to internal levels of actin and are averaged from at least three separate cortices per genotype (n= 3-6).

At E12.5 (A), an increase was seen in the levels of E2f8 with loss of Rb or dual loss of p107 and p130, although levels of E2f8 were at the threshold of detection at E12.5, as determined by large Ct values. At E14.5 (B), there were modest changes in E2f family members. Notable changes are in levels of E2f1 with loss of Rb (Student’s t-test p= 0.0009) and in E2f4 with loss of p107 (Student’s t-test p= 0.0013).
increase in E2f1 expression in the Rb deficient cortex could lead to increased proliferation, and therefore an increase in Rbp-LacZ expressing neurons, the changes in E2f levels within the p107-/-;p130-/- cortex are minimal and are unlikely to explain a decrease in Rbp-LacZ activity.

p53 family member expression in cortex lacking various Rb family members

As discussed earlier, E2F family members are known to control key regulators of the apoptotic pathway including p73, Noxa, and Puma. Therefore, loss of Rb family members may lead to deregulation of E2F target genes and thus apoptosis within the developing cortex, which could indirectly lead to changes in Rbp-LacZ activity. To test this, the mRNA levels of p53 family members as well as key E2F targets (Noxa, Puma Mcl1) that regulate apoptosis were analyzed on the different Rb family deficient backgrounds in the E12.5 brain and E14.5 cortex. Loss of Rb led to very modest changes in levels of apoptotic regulators with only a small increase in p63 levels at E14.5 (Table 3-5). Loss of p107, p130, or dual loss of p107 and p130 did not lead to any significant changes in p53 or p73 in the E12.5 brain or E14.5 cortex. Interestingly, at E12.5 there was an increase in the level of p63 with loss of p107 and a decrease in the level of p63 with dual loss of p107 and p130. At E12.5, the level of p63 in the brain was very low, leading to high variability between samples, and so these changes were not statistically significant. Since this change was not seen again at E14.5, where the level of p63 is greater and when analysis of Rbp-LacZ expression was evaluated within the embryo, this difference was not investigated further. Expression of this subset of apoptotic regulators are not deregulated with loss of Rb family members in the E14.5 cortex, and thus cannot explain the differences seen in Rbp-LacZ activity within the embryo.

Summary

The changes in proliferation and apoptosis seen in the Rb deficient cortex were similar to what has been reported by other laboratories (L. Wu et al., 2003; MacPherson et al., 2003; Ferguson et al., 2005). The increase in BrdU incorporation and increase in cortical size (as judged by changes in cortical area) both suggest that the Rb deficient cortex may contain more Rbp-LacZ expressing neurons and therefore, while proportionally the same number of cells are LacZ positive, it would appear that there is an increase in Rbp-LacZ activity. Alternatively, the increase in proliferation with Rb loss may lead to an increase specifically in the number of Rbp-LacZ expressing cells. An increase in E2f1 levels in the cortex
### Table 3-5: Minimal deregulation of p53 family members with loss of Rb family members.

Changes in mRNA levels of p53 family members and other regulators of apoptosis in the various Rb family member deficient cortices is given as the ratio of the target copy number in the mutant cortex divided by the target copy number of the respective wild type cortex. Target copy numbers are normalized to internal levels of actin and are averaged from at least three separate cortices per genotype (n=3-6). At E12.5 (A), changes were seen in levels of p63 in p107/- cortex and p107-/-;p130-/- cortex but these results were not significant due to large variation between samples. At E14.5 (B), there were only moderate changes in p53 family members.

<table>
<thead>
<tr>
<th></th>
<th>Nes-Cre;RbLoxP/LoxP</th>
<th>p107-/ RbLoxP/LoxP</th>
<th>p130-/ RbLoxP/LoxP</th>
<th>p107-/-;p130-/- RbLoxP/LoxP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>p53</em></td>
<td>0.97</td>
<td>1.07</td>
<td>1.01</td>
<td>1.06</td>
</tr>
<tr>
<td><em>p63</em></td>
<td>1.54</td>
<td>1.03</td>
<td>1.20</td>
<td>0.85</td>
</tr>
<tr>
<td><em>p73</em></td>
<td>0.89</td>
<td>1.01</td>
<td>1.30</td>
<td>1.16</td>
</tr>
</tbody>
</table>

#### E12.5 Brain

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rb-/- Rb+/-</th>
<th>p107-/ p107+/-</th>
<th>p130-/ p130+/-</th>
<th>p107-/-;p130-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p53</em></td>
<td>1.09</td>
<td>1.11</td>
<td>1.06</td>
<td>1.04</td>
</tr>
<tr>
<td><em>p63</em></td>
<td>0.82</td>
<td>8.44</td>
<td>0.78</td>
<td>0.18</td>
</tr>
<tr>
<td><em>p73</em></td>
<td>1.06</td>
<td>0.84</td>
<td>0.89</td>
<td>0.89</td>
</tr>
<tr>
<td><em>Noxa</em></td>
<td>1.08</td>
<td>1.13</td>
<td>0.88</td>
<td>1.01</td>
</tr>
<tr>
<td><em>Puma</em></td>
<td>0.89</td>
<td>0.99</td>
<td>0.95</td>
<td>1.04</td>
</tr>
<tr>
<td><em>Mcl1</em></td>
<td>0.98</td>
<td>0.99</td>
<td>1.04</td>
<td>0.99</td>
</tr>
</tbody>
</table>
is consistent with the observed increase in proliferation within the Rb cortex. While E2F1 is the primary E2F involved in activating cell death, there was no increase in cell death with conditional loss of Rb, consistent with what was previously reported (Ferguson et al., 2002; MacPherson et al., 2003). Whether these changes in proliferation and E2f family member expression contribute to the lethality of the Rb-deficient embryo, due to disruption of cortical development, is unclear since neuronal specific loss of Rb also leads to defects in neuronal migration, which we have not analyzed (Ferguson et al., 2005). Although these data suggest that the increase in RbP-LacZ activity could be indirectly due to increased proliferation, the fact that pRB can be found at the Rb promoter suggests that loss of Rb could cause a direct de-repression of the Rb promoter.

The double knockout of p107 and p130 did result in a small increase in proliferation when compared to an in-litter control, but since the control is deficient in p130, this level of proliferation is not greater than in a normal cortex. There was also no ectopic proliferation in the p107-/-;p130-/- cortex, which was seen with Rb loss, further supporting the idea that loss of Rb and dual loss of p107 and p130 affect neuronal development differently. Loss of p107 and p130 did lead to a further decrease in apoptosis within the cortical plate and intermediate zone of the cortex, indicating that the apoptotic pathway is disturbed. It has been previously reported that loss of p130 on an enriched Balb/c background leads to increased apoptosis within the neural tube and floor plate at E10.5 in a strain specific manner (LeCouter et al., 1998b). Apart from the difference in strain, this data could be due to differences in developmental time point or the site of analysis, particularly since p130-/- embryos on a Balb/cJ background die between E11 and E13.5, before corticogenesis, which therefore precludes study at later time points.

While there did not appear to be differences in p53 family members at the mRNA level in E14.5 cortex, a more intensive investigation of the apoptotic factors would be needed to determine the mechanism. For example, we did not analyze levels of the E2F target p19ARF, which can increases the activity of p53 and p73 at the protein level by negatively regulating MDM2 (Polager and Ginsberg, 2008). A decrease in apoptosis cannot explain the decrease in RbP-LacZ activity; in fact, one would expect more RbP-LacZ activity with less apoptosis. However, if there is less cell death occurring within the population of cells that do not express RbP-LacZ, this might lead to an overall decrease in the proportion of RbP-
LacZ expressing cells within the cortex, and consequentially, decreased appearance of RbP-LacZ activity.

The binding of pRB family members at the Rb promoter suggests that Rb family members directly regulate Rb expression. However, the loss of various Rb family members also led to many indirect changes in neuronal development that may account for the changes seen in RbP-LacZ activity in the cortex. To differentiate between these possibilities, we need to determine if the change in RbP-LacZ activity with Rb family deficiency occurs in a cell autonomous or non-cell autonomous manner.
Chapter 4: *RbP-LacZ* Activity in Primary Cortical Culture with Loss of Various *Rb* Family Members

Development of a Cortical Neuronal Culture System

- Plating embryonic cortical neurons without FGF
- Timecourse of proliferation

Identification of Cell Populations

- Identity of progenitor populations
- Proliferation in progenitor populations
- Identity of mature neuronal populations

*RbP-LacZ* Activity in Neuronal Culture

- *LacZ* activity in wild type T157 cultures
- *LacZ* Activity in various *Rb* family deficient T157 cultures

Proliferation in Various *Rb* Family Deficient T157 Cultures

- Analysis of proliferation in *Rb* family mutant cultures
- Proliferation in *Rb*-deficient progenitors

Comparison of Neuronal Populations in Various *Rb* Family Deficient Cultures

- Maintenance of neurogenesis in *Rb* family mutant cultures
- Analysis of mature neuronal subtypes in *Rb* family mutant cultures
- Analysis of neuronal progenitors in *Rb* family mutant cultures
  - Regulation of Pax6 by *Rb* family members

Identification of *RbP-LacZ* Expressing Cells

- *RbP-LacZ* activity and neuronal maturity
- *RbP-LacZ* activity in neuronal progenitors

*RbP-LacZ* Activity in Subpopulations with Loss of Various *Rb* Family Members

- *p107*- or *p130*- deficient cultures
- *p107* and *p130*- deficient cultures
  - Changes in frequency of Pax6+ *RbP-LacZ* expressing cells
  - Changes in intensity of *RbP-LacZ* in Pax6+ cells
- *Rb* deficient cultures
  - Changes in *RbP-LacZ* activity in neuronal subpopulations
  - Changes in nuclear size with *Rb* loss

Summary
Development of a Primary Cortical Culture from the T157 RbP-LacZ E14.5 Cortex

The activity of the RbP-LacZ reporter (T157) was deregulated in both Rb-deficient and p107; p130 doubly deficient cortex, but the loss of these Rb family members had opposing effects on RbP-LacZ activity. To determine if the deregulation of RbP-LacZ with loss of Rb family members in the cortex is due to a cell autonomous or a non-cell autonomous effect, it was necessary to look at the expression of RbP-LacZ on a cell-by-cell basis. Therefore, it was decided to analyze RbP-LacZ activity in dissociated primary cortical neuron culture, which provides a mechanism to examine both the intensity of RbP-LacZ activity within each cell as well as the number of RbP-LacZ expressing cells within the culture. Primary neuron culture also provides a method to identify the cell types exhibiting RbP-LacZ activity. The changes seen in RbP-LacZ activity within the cortex with loss of various Rb family members was regionally restricted within the cortex, and this distribution could represent the enrichment of a specific neuronal cell type. Therefore, the intensity, frequency, and identity of the RbP-LacZ expressing cells were monitored in primary cortical neuron culture to determine if Rb family members deregulate RbP-LacZ activity in a cell autonomous manner versus non-cell autonomous manner.

Generation of a 2-dimensional neuronal specific culture

Primary cortical neuron cultures were generated from E14.5 micro-dissected cortices by using enzymatic digestion and mechanical disruption to create single cell suspensions (Figure 4-1 A) (Hutton and Pevny, 2008). These were then plated on poly-D-lysine / laminin treated coverslips and grown inverted in defined Neurobasal medium with B27 supplement, containing a cocktail of factors which has been shown to encourage the development of neuronal lineages (Brewer et al., 1993). Dissociated cells from E14.5 embryonic cortices tend not to attach with high affinity to the poly-D-lysine / laminin coverslips, and instead, grow as neurospheres, non-adherent spherical clusters of cells that are generated from proliferating progenitor cells (Reynolds and Weiss, 1992). The tendency of dissociated cells to grow aggregated in suspension precluded analysis of RbP-LacZ activity, but neuronal outgrowth of adherent cells was observed initially on the underside of coverslips in these neurosphere laden cultures. Therefore, to encourage two dimensional and adherent neuronal outgrowth, dissociated cells were allowed to attach to poly-D-lysine / laminin coverslips first, and then these coverslips were inverted within the culture media. We selected Neurobasal media because it encourages the growth of neurons over other cell types (e.g.
Figure 4-1: Establishment of E14.5 primary cortical neuron culture.

The protocol for processing E14.5 cortex for culture is shown in (A). Single cell suspensions derived from embryonic cortex were plated on coverslips and allowed to attach for 4 hours before inverting the coverslip. An example of the morphological changes in C57BL/6 wild type neuron culture is shown in (B). The development of elongated neuronal cell bodies can be seen in Day 4 and Day 6 cultures.
neuronal cell survival without adding FBS or FGF, both of which stimulate proliferation of neuronal progenitors (Lukaszewicz et al., 2002; Iwata and Hevner, 2009). After one day in culture, most cells beneath the coverslip were still very rounded but neurite outgrowths were seen by microscopic analysis. By Day 2 in culture, most cells had obvious projections (Figure 4-1 B), and as time in culture increased, the length and extent of branching of these projections increased so that by Day 4 in culture, many cells had long axonal projections. By Day 6 in culture, complex networks of intertwined projections between cells could be seen growing in culture, corresponding to morphological changes expected with neuronal differentiation.

Characterization of proliferation in E14.5 primary cortical cultures

While morphologically the primary neuron culture appeared to contain many mature neurons, it was important to see if, similar to within the embryo, there were both proliferating progenitor populations as well as developed neurons. Additionally, with increased differentiation over time, one might expect there also to be concomitant changes in proliferation. To test if this was the case, proliferation in primary cortical neuron culture was monitored by EdU (5-ethynyl-2'-deoxyuridine) incorporation. The benefit of using EdU incorporation (as opposed to BrdU incorporation) is that it does not require the acid-based denaturation of DNA, allowing for the maintenance of the delicate cell structure of neurons. The deoxyuridine analog, EdU, contains an alkyne group, which after incorporation into DNA and cell fixation, reacts with an azide-modified AlexaFluor dye to indicate proliferating cells. Cultures were pulsed for 2 hours with EdU and then analyzed for proliferation on Days 2 and 4. Under these culture conditions (Neurobasal medium with B27 supplement but no FGF), proliferation remained at a low rate through 6 days in culture, with a small decrease in proliferation by Day 6 (Figure 4-2).

Identification of Diverse Populations in Primary Cortical Neuron Cultures

Progenitor subpopulations in primary cortical neuron cultures

Now that it was established that there is a proliferating population within these culture conditions, it was next important to know if these cells represent the progenitors within the developing cortex. The developing cortex contains two main types of progenitor cells: apical and basal progenitors. Apical progenitors consist initially of neuroepithelial cells which give rise to radial glial cells in mice by E10. Apical progenitors undergo M phase along the apical ventricular surface, and undergo either asymmetric
Figure 4-2: A low level of proliferation is maintained in E14.5 cortical neuron culture. Proliferation was measured in cortical neuron culture by pulsing cells with EdU for 2 hours. A small population of cells was proliferating on Days 2, 4, and 6 in culture (A). Quantification of the percentage of EdU positive cells for all experiments is shown in panel (B). N = number of DAPI cells analyzed. Day 2 and Day 4, n= 14 embryos; Day 6, n= 8 embryos

<table>
<thead>
<tr>
<th></th>
<th>Day 2</th>
<th>Day 4</th>
<th>Day 6</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edu +</td>
<td>4.4 ± 0.3%</td>
<td>5.0 ± 0.4%</td>
<td>2.9 ± 0.1%</td>
<td>900-1300</td>
</tr>
</tbody>
</table>
divisions (which give rise to one radial glial cell and one post-mitotic neurons) or symmetric divisions (which give rise to two basal or intermediate progenitors). The basal progenitors divide within the subventricular zone and do not have contact with the ventricular surface (reviewed in Pinto and Gotz, 2007). Intrinsic cell markers have been used to identify different subtypes of progenitors. Sox2 is one of the earliest transcription factors expressed in the developing central nervous system (CNS) and is important for the maintenance and proliferation of apical neuronal progenitors. Sox2 is highly expressed within the ventricular zone of the developing cortex and loss of Sox2 leads to impaired neuronal differentiation (Bani-Yaghoub et al., 2006; Cavallaro et al., 2008). Loss of Sox2 also leads to a depletion of neuronal progenitors within the areas of adult neurogenesis, the dentate gyrus and subventricular zones (Pevny and Nicolis, 2010). Primary neuron cultures were examined for Sox2 expressing progenitors in Day 4 cultures (Figure 4-3 B); 22.8 ± 3.3% of primary neuron culture is positive for Sox2, with 20.3 ± 4.0% of the positive cells showing an intense Sox2 nuclear signal by immunocytochemistry (n = 6 embryos).

Nestin is an intermediate filament expressed in the precursor of both neuronal and glial cells, and is downregulated upon neuronal differentiation (Dahstrand et al., 1995; Gilyarov, 2008). It is considered to be a marker of neural stem cells because of its expression both in the proliferating cells of the developing cortex and the adult neurogenic niches. Nestin is also expressed in mature astrocytes, which should not be found in the E14.5 cortex. In Day 4 cultures, 18.5 ± 4.2% of the culture is positive for Nestin (Figure 4-3 F).

Pax6 is a paired-box transcription factor that marks radial glial cells within the ventricular zone of the developing cortex that have a neurogenic fate. Pax6 is required for proper cell cycle exit of dividing cells as well as appropriate development of cortical neurons (Quinn et al., 2007; Georgala et al., 2011b). Loss of functional Pax6 occurs in Pax6 (Sey/Sey) mutant mice and causes lethality by birth, and these mice display a malformed cerebral cortex and the absence of eyes (reviewed in Georgala et al., 2011b). Using a rabbit polyclonal to Pax6 that recognizes C-terminal region, 8.9 ± 0.7% of wild type culture was Pax6+, with a small percentage expressing high levels (14.2 ± 1.1% of positive cells) (Figure 4-3 C). These results were repeated with a mouse monoclonal antibody specific to the N-terminus of Pax6, in
Figure 4-3: Various neuronal progenitors are found in E14.5 cortical neuron culture on Day 4. Examination of neuronal progenitors in Day 4 cortical neuron culture was performed by immunocytochemistry. A rabbit polyclonal antibody to pro-hormone convertase (PC1/3) was used as a negative control for polyclonal antibodies (pink). Panel (G) shows percentages of each marker in Day 4 culture from multiple experiments except for Tbr2*, which was done on Day 3. n= number of embryos analyzed; N = 400 -800 DAPI cells analyzed.
which 8.9±1.0% of cells were positive for Pax6, and 18.3 ± 2.3% of the positive cells showed strong expression.

Pax6-positive radial glial cells can give rise to basal progenitors, which are more committed to a neuronal fate than apical progenitors, and only undergo symmetric divisions to form two post mitotic neurons. Tbr2 is a marker of these basal progenitor cells, which are found within the sub-ventricular zone of the developing cortex (Englund et al., 2005, Hevner et al., 2006). On Day 4 in culture, most of the cells showed some low level of perinuclear Tbr2 staining. Therefore, Tbr2 counts were done at Day 3 where there was a greater differential between positive and negative cells as well as a more intense nuclear stain. On Day 3 in culture, 30.7 ± 3.2% of cells expressed nuclear Tbr2 (Figure 4-3 D). Therefore, there are subpopulations of progenitor cells within E14.5 primary cortical cultures that represent different levels of commitment towards neuronal differentiation.

**Proliferation in neuronal progenitor populations**

To investigate whether these progenitor markers were indeed marking the proliferating population, Day 4 cultures were pulsed with EdU for 2 hours and stained for EdU incorporation and for the presence of these markers using immunocytochemistry. We found that both Sox2 and Pax6 progenitor cells are proliferating in primary cortical neuron culture at Day 4 (Figure 4-4). The majority of EdU+ cells were also positive for Sox2 (78.8%), and this represented 36.3% of the Sox2 population, confirming that Sox2 expression is indeed expressed within dividing progenitors similar to the situation within the cortex. A smaller percentage of EdU+ cells were positive for Pax6 (44.2%) using a rabbit polyclonal antibody, representing 34.5% of the Pax6+ population. Similar results were seen with a mouse monoclonal to Pax6 (data not shown). Nestin was expressed in all EdU+ cells (Figure 4-5), representing 40.9% of Nestin positive cells. NeuN, a marker of differentiated neurons (Mullen et al., 1992), did not stain any EdU+ cells, confirming that it does not mark neuronal progenitors (Figure 4-5). Therefore, neural progenitors within the primary cortical culture system have features similar to those displayed within the developing cortex.

**Mature neuronal populations in primary cortical cultures**

The E14.5 cortex represents the midpoint of neuronal corticogenesis, which begins at around E10 and lasts until E18, when gliogenesis begins. The cortex is comprised both of projection neurons, which
Figure 4-4: Pax6 and Sox2 progenitors are proliferating in cortical neuron culture on Day 4. Wild type C57BL/6 cultures were pulsed for 2 hours with EdU, followed by immunocytochemistry for Pax6 and Sox2. A percentage of both Pax6 (Row B) and Sox2 (Row C) positive cells were proliferating on Day 4. Double stained cells are shown in yellow. Panel (D) shows the fraction of overlap between marker (Pax6 and Sox2) and EdU positive cells. N = total number of DAPI cells analyzed.
Figure 4-5: Nestin progenitors are proliferating in cortical neuron culture on Day 4.

Wild type C57BL/6 cultures were pulsed for 2 hours with EdU followed by immunocytochemistry for Nestin and NeuN. A fraction of Nestin positive cells (Row B) were positive for EdU while no NeuN positive cells (Row C) were found to be EdU positive. Double stained cells are shown in yellow. Panel (D) shows the fraction of overlap between marker (Nestin and NeuN) and EdU positive cells. N= total number of DAPI cells analyzed.
have been derived from the pallium and migrate radially from the ventricular zone into the upper cortical layers in a inside-out manner, as well as interneurons generated within the subpallium which migrate tangentially into the developing cortex (See Figure 3-7). These different types of neurons can be distinguished by the presence of various markers.

First, to establish that the majority of the primary cortical culture is indeed neuronal, cells were immunostained with markers for identifying neurons at different levels of maturity (Figure 4-6). Class III β-tubulin is one of the first neuronal-specific cytoskeletal proteins expressed after the terminal mitosis of differentiating neurons (Menezes and Luskin, 1994; M.K. Lee et al., 1990). The majority of cells in the E14.5 cortical culture stain positive for β-tubulin III (TUJ1) (93.5 ± 1.0%) in Day 4 culture (Figure 4-6 B). Map2a and Map2b are two neuronal specific isoforms of microtubule-associated proteins expressed only in differentiated neurons (Dehmelt and Halpain, 2005). Their expression follows the appearance of β-tubulin III staining and Map2b is maintained within adult neurons. The majority of cells in the E14.5 cortical culture are Map2a,b positive (93.7 ± 1.0%) in Day 4 cultures (Figure 4-6 C). NeuN is a neuron-specific nuclear protein that marks post-mitotic neurons (Mullen et al., 1992), and its expression is maintained within mature neurons. Although used for many years as a general marker of neurons, it has recently been identified as the neuronal specific splicing factor Fox-3 (Kim et al., 2009). In Day 4 cortical cultures, 36.5 ± 2.1% of cells express NeuN (Figure 4-6 D). If most of the culture expressed neuronal specific markers, then one would expect very small amounts of the two main glial cell types, astrocytes and oligodendrocytes. Indeed, when cultures are investigated for the markers for astrocytes, GFAP, and oligodendrocytes, O4, there are very low numbers of these populations. In Day 4 cultures only 2.2 ± 0.35% of cells are positive for GFAP (Figure 4-6 E). O4, an early marker of oligodendrocytes (Miller, 2002) was not consistently present in Day 4 cultures, with only 0.7 ± 0.3% O4+ cells. At Day 6, finding O4+ cells was more reliable, with 2.1 ± 0.3% of cells positive for O4 (Figure 4-6 G). Overall, the majority of cells are of a neuronal lineage in these defined growing conditions.

Projection neurons and interneurons can be differentiated by which neurotransmitters they express; projection neurons are primarily glutamatergic and interneurons are GABAergic. The majority of the developing cortex consists of glutamatergic neurons while interneurons make up only 20% of the total neurons (reviewed in Barinka and Druga, 2010). One marker of GABAergic neurons is glutamate
Figure 4-6: Primary cortical culture consists of predominately neuronal cell types on Day 4. Wild type primary cortical cultures were examined on Day 4 by immunocytochemistry for the presence of neuronal or glial markers. While most cells stained positive for neuronal markers Tuj1 (B), Map2a,b (C), and NeuN (D), only a small percentage of cells expressed glial markers GFAP (E) or O4 (G). Quantification of the percentages of each cell type over multiple experiments on Day 4 is shown in panel (I). Staining for RbP-LacZ activity of the same culture shows Xgal positivity on Day 4 (H). The staining for O4 was moved to Day 6 due to the low frequency on Day 4. n= number of embryos analyzed; N= 300-600 DAPI cells analyzed.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Marker +</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tuj1</td>
<td>93.5 ± 1.0%</td>
<td>5</td>
</tr>
<tr>
<td>Map2a,b</td>
<td>93.7 ± 1.0%</td>
<td>8</td>
</tr>
<tr>
<td>NeuN</td>
<td>36.5 ± 2.1%</td>
<td>3</td>
</tr>
<tr>
<td>GFAP</td>
<td>2.2 ± 0.3%</td>
<td>3</td>
</tr>
<tr>
<td>O4</td>
<td>0.7 ±0.4%</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 4-7: A variety of mature neuronal subtypes are present in cortical neuron culture on Day 4. Wild type primary cortical cultures were examined on Day 4 by immunocytochemistry for the presence of different mature neuronal subtypes. A percentage of GABAergic neurons were detected by presence of GAD67 (C). A small percentage of Reelin (B), Calretinin (E), or Calbindin (G) positive neurons were also detected in the same Day 4 culture. Quantification of percentages of each cell type is shown in panel (H). n= number of embryos analyzed; N= 500-1000 DAPI cells analyzed.
decarboxylase, GAD67, which converts glutamate to GABA. In Day 4 cultures 14.0 ± 0.7% of cells express GAD67 (Figure 4-7 C), consistent with GABAergic neurons making up just a small proportion of the developing cortex. Another way neurons are distinguished is by the expression of the calcium binding proteins, calretinin or calbindin, whose expression is mutually exclusive (reviewed in Barinka and Druga, 2010). The majority of calretinin and calbindin expressing cells are also GABAergic interneurons and are derived from the subpallium (Barinka and Druga, 2010). Small percentages of calretinin+ (4.6 ± 0.4%) and calbindin+ (5.5 ± 0.5%) stain positive in Day 4 cultures (Figure 4-7 E and G). Cajal–Retzius cells are a subset of calretinin+ cells that are glutamatergic and express the reelin marker (Barinka and Druga, 2010). These are some of the earliest born neurons, appearing as early as E10. Cajal-Retzius neurons are found within the outermost marginal zone of the embryonic cortex and they secrete reelin, an extracellular matrix protein essential for the proper migration and lamination of the cortex (Z. Huang, 2009). Reelin+ cells make up 3.2 ± 0.2% of Day 4 cultures (Figure 4-7 B).

The diverse cell types within primary cortical culture are representative of the different cell types that one would find within the developing cortex. However, the predominance of neurons versus glial cells is more similar to the situation before E18 when gliogenesis commences. Developing a culture system that is similar to what is seen in vivo allows for cell-by-cell analysis of RbP-LacZ activity in Rb family deficiencies.

Analysis of RbP-LacZ Activity in E14.5 Primary Cortical Neuron Cultures

RbP-LacZ activity in wild type T157 cortical cultures

To determine the baseline activity of the RbP-LacZ transgene in primary cortical neuron cultures, wild type T157 cultures were stained with Xgal on Day 2, Day 4, and Day 6 (Figure 4-8 A). Initial experiments were done using the conventional method of Xgal staining for LacZ activity, using a final concentration of 5mM K₃Fe(CN)₆ and 5mM K₄Fe(CN)₆, and only a low level of RbP-LacZ activity was detected in primary cortical cultures, even after overnight staining. To overcome the low Xgal reactivity, we optimized a new staining protocol to increase Xgal reactivity, which used 1mM K₃Fe(CN)₆ and 1mM K₄Fe(CN)₆. This modification increased the strength of the Xgal signal both in frequency and intensity, and allowed for shorter staining times, which helped maintain neuronal morphology and structural integrity. We found strong Xgal reactivity after only 4 hours of staining of Day 6 cultures, as opposed to
Figure 4-8: **RbP-LacZ** activity increases in frequency and intensity with time in culture. 

*RbP-LacZ* activity in T157 primary cortical cultures was examined by overnight Xgal staining. Panel (A) shows *RbP-LacZ* activity in T157 culture on Days 2, 4, and 6. An increase in both the frequency (Total Xgal+/Total Cells) and intensity (Strong Xgal+/Total Xgal+) of *RbP-LacZ* activity was seen with time in culture. Panel (B) shows the quantification of frequency (Xgal+) and intensity (Strong Xgal+) over multiple experiments. The increase in frequency (C) and intensity (D) of Xgal staining from Day 2 to Day 6 is statistically significant. (Frequency: student's t-test $p = 0.0006$; Intensity: student's t-test $p = 0.011$; $n= 5$ embryos, $N= 500-1000$ cells/experiment)
the greater than 8 hours required using the older protocol. All subsequent Xgal staining of primary cortical cultures was performed with this optimized protocol. Frequency of Xgal+ cells was defined as the percentage of Xgal+ cells within the total culture, and intensity of Xgal+ cells was defined as the percentage of Xgal+ cells within the Xgal+ population that displayed saturating levels of Xgal, defined as the overexposure of Xgal stain as determined by visual inspection of manipulated images (as described in Methods).

Even though a microdissected T157 cortex at E14.5 shows strong LacZ activity within an hour of staining in Xgal, only 10.7 ± 3.1% of primary cortical cells express the RbP-LacZ transgene after 2 days in culture, and only 10.5 ± 4.5% of the Xgal+ cells displayed an intense blue stain. Interestingly, as time in culture progressed, there was a significant increase in the percentage of cells with RbP-LacZ activity (Figure 4-8 C). By Day 6 in culture, 24.8 ± 6.2% of T157 cells show RbP-LacZ activity, representing a statistically significant increase from the activity measured on Day 2 (Student’s t-test, \( p = 0.0006 \)). Additionally, the intensity of Xgal+ cells also increased significantly from Day 2 to Day 6, from 10.5 ± 4.5% to 25.1 ± 5.2% of the Xgal+ population (Student’s t-test, \( p = 0.011 \)) (Figure 4-8 D). Analysis of RbP-LacZ activity on Day 4 showed modest increases compared to that on Day 2. This indicates that as the morphology of primary cortical cultures progresses to a more mature and differentiated neuronal state, there is an increase in the frequency and intensity of RbP-LacZ observed. This agrees with several studies showing that increases in neuronal differentiation lead to increases in Rb expression (Gill et al., 1998; Slack et al., 1993). We have observed that not all differentiated neurons were positive for RbP-LacZ activity, suggesting that Rb expression is not just increased in all mature neurons, but may be expressed in a temporal- or lineage-specific manner within the developing cortex.

RbP-LacZ activity in primary cortical cultures lacking various Rb family members

To determine how loss of various Rb family members would impact RbP-LacZ activity in primary cortical cultures, RbP-LacZ activity was monitored in primary cortical cultures that were established from mutant E14.5 embryos lacking various Rb family members, and carrying the T157 transgene. Cortices from E14.5 embryos resulting from T157 mutant crosses were dissociated, and midbrains were stained with Xgal to identify those embryos carrying the T157 transgene, which were subsequently plated. Genotyping of the inner yolk sacs (as well as midbrains for Rb crosses) revealed which embryos bore the
desired wild type and mutant genotypes for various Rb family members. On Day 2, Day 4 and Day 6, primary cortical cultures from the subset of wild type and mutant embryos bearing the T157 transgene were stained with Xgal and scored to determine the frequency and intensity of RbP-LacZ activity (Figure 4-9).

Primary cortical neuron cultures from T157; p107+/+ and T157;p107-/- E14.5 embryos were processed for Xgal staining on Days 2, 4, and 6 and there was no significant difference in RbP-LacZ activity between these genotypes, consistent with the results of whole mount analysis (Figure 2-1). Likewise, primary cortical neurons from T157; p130+/+ and T157; p130-/- E14.5 embryos displayed no significant difference in RbP-LacZ activity between T157; p130+/+ or T157; p130-/- cultures, in agreement with the small decrease or no change in RbP-LacZ activity observed in whole mount embryos or cortex with loss of p130 (Figures 2-2 and 2-11).

Next, RbP-LacZ activity was analyzed in primary cortical neuron cultures from T157; p107-/-; p130-/- and T157; p107+/+; p130-/- E14.5 embryos. Out of three separate experiments, only one showed a decrease in RbP-LacZ activity on Days 2, 4, and 6 with loss of both p107 and p130. There was a small decrease in the percentage of RbP-LacZ positive cells on Day 6 in culture, but overall the decrease in RbP-LacZ activity with combined loss of p107 and p130 was not statistically significant (Figure 4-9). Thus, the consistent decrease in RbP-LacZ activity observed in the analysis of whole mount embryos (Figure 2-3) and micro-dissected cortices (Figure 2-12) was not obvious in analysis of primary cortical cultures. The basis for this discrepancy has been addressed in experiments described in later sections of this Chapter.

Whether examined in whole mount embryos or in micro-dissected brains, conditional loss of Rb led to an increase in RbP-LacZ activity within the cortex at E14.5 (Figures 2-8 and 2-13). To test whether this could be replicated in primary cultures, we plated wild type (T157;RbLoxP/+ or T157;RbLoxP/LoxP) and Rb-deficient (T157;NestinCre;RbLoxP/LoxP) primary cortical neurons and monitored RbP-LacZ activity by Xgal staining on Days 2, 4, and 6. When comparing T157;NestinCre;RbLoxP/LoxP cultures to either T157;RbLoxP/+ or T157;RbLoxP/LoxP cultures, there was no obvious difference in RbP-LacZ activity (Figure 4-9) (percentage of Xgal+ cells or the percentage of cells with intense Xgal staining, as judged by criteria described in Methods).
Figure 4-9: No significant changes in *RbP-LacZ* activity in the various *Rb* family deficient cortical neuron cultures. The frequency (Column A) and intensity (Column B) of Xgal positive cells in cortical cultures derived from various *Rb* family deficient embryos were analyzed on Days 2, 4 and 6. For all examined genotypes, there were no statistically significant changes in *RbP-LacZ* activity. Graphs show averages of frequency and intensity for wild type (solid) and *Rb* family deficient (striped) cultures. n= 2-4 embryos per genotype; N= 600-900 cells per experiment.
Therefore, it appears at first glance that in primary cortical cultures, the changes seen in Rb-P-LacZ activity with loss of Rb family members within the embryo were not replicated in tissue culture. One reason for this discrepancy may be that the changes seen in Rb-P-LacZ activity within the embryo may be specific to a subset of cells that are clustered within the developing cortex, and by analyzing the total primary culture, in which these cells are dispersed, the effect on Rb-P-LacZ activity is diminished or lost. This notion is consistent with the results from whole mount Xgal staining of the cortex (Figures 2-12 and 2-13), showing loss of Rb or dual loss of p107 and p130 led to a regional-specific change in Rb-P-LacZ activity. The assumption is that if the cells that resided in those LacZ+ regions could be identified, then changes in the frequency or intensity of the LacZ+ signal within the populations that normally express LacZ in primary cortical culture might be consistent with changes seen in the whole embryo (cell-autonomous effects). A second possible reason for the discrepancy in Rb-P-LacZ activity in embryos compared to cortical culture is that specific interactions between neurons and spatial cues (non-cell autonomous effects) necessary for the changes seen in the intact embryo or brain are destroyed in two-dimensional tissue culture models. Alternatively, tissue culture conditions may be altering Rb expression, as represented by Rb-P-LacZ expression, due to differences in cell to cell signaling caused novel cell to cell contacts and/or changes in cell cycle dynamics (such as the onset of senescence). Finally, it could be that the loss of Rb family members affects proper neuronal migration/lamination, and that it is not the frequency or intensity of cells with Rb-P-LacZ expression that is changing, but rather their ability to be in the right place at the right time during development.

**Effect of Rb Family Member Loss on Proliferation in Primary Cortical Cultures**

Proliferation in E14.5 wild type cortical cultures remains very low and remains relatively constant over time in culture (Figure 4-2). When levels of proliferation were analyzed in the E15 embryo, loss of p107 or p130 alone led to a small decrease in BrdU positive cells, while dual loss of p107 and p130 returned proliferation to normal levels. To test if there were any changes in proliferation with loss of p107, p130, or dual loss of p107 and p130, primary cortical cultures from wild type or mutant embryos were pulsed for 2 hours with EdU before harvest on Days 2 and 4, and proliferation was measured. However, we found no significant changes in the percentage of EdU positive cells with loss of p107 or p130 or both p107 and p130 on Days 2 or 4 in culture (Figure 4-10).
Figure 4-10: Loss of *Rb* leads to increased proliferation in cortical neuron culture on Day 2. Proliferation levels were measured in the various *Rb* family member deficient backgrounds by pulsing cultures for 2 hours with EdU and counting the number of EdU+ cells relative to DAPI. There is a significant increase in proliferation in Day 2 *NestinCre;RbLoxP/LoxP* cortical cultures relative to *RbLoxP/LoxP* cultures (student’s t-test $p=0.0004$). Graphs show averages for control (solid) and the various *Rb* family member deficient (striped) cultures. n= number of embryos analyzed; N= 1000-1500 cells per experiment.
Figure 4-11: Pax6 progenitors are proliferating in Rb-deficient cortical cultures on Day 2.

Primary cortical cultures derived from RbLoxP/+ and NestinCre;RbLoxP/LoxP embryos were pulsed for 2 hours with EdU on Day 2. EdU+ Pax6+ progenitors could be found in both wild type (A) and Rb-deficient (B) cultures. There was a small increase in the number of Pax6+ progenitors within the proliferating populations with Rb deficiency (C). Co-stained cells are shown in yellow.   N= number of DAPI cells analyzed.
*Rb*-deficient E15 embryos showed ectopic proliferation outside of the ventricular and subventricular zone (Figure 3-10). To test if increased proliferation could be seen in primary cortical cultures, wild type (*Rb*LoxP/+ or *Rb*LoxP/LoxP) and *Rb*-deficient (*NestinCre;Rb*LoxP/LoxP) cultures were pulsed with EdU for 2 hours before harvest on Days 2 and 4 and percentages of EdU+ cells were monitored. In Day 2 cultures, there was a 70% increase in EdU+ cells in *Rb*-deficient culture compared to wild type (Student’s t-test \( p = 0.0004 \)) (Figure 4-10). By Day 4 in culture, there no longer is a difference in proliferation between wild type and *Rb*-deficient cultures. This increased proliferation with loss of *Rb* on Day 2 is consistent with the increased proliferation seen in the E15 embryo using BrdU incorporation; however, it does not correlate with changes in *RbP*-LacZ activity in *Rb*-deficient cultures (Figure 4-9).

Loss of *Rb* alone, but not loss of *p107*, *p130*, or *p107* and *p130*, led to increased proliferation in Day 2 cultures. Therefore, it was of interest to see if loss of *Rb* changes the proliferative index of neuronal progenitors. Day 2 cultures received a 2-hour pulse of EdU before harvest, and then were assayed for EdU incorporation, followed by immunocytochemical detection of Sox2 or Pax6 (Figure 4-11). We found that both Pax6 and Sox2 progenitors are proliferating in *NestinCre;Rb*LoxP/LoxP cultures. There was a modest increase in the percent of Edu+ cells scoring positive for Pax6 (wild type, 28.6% vs. *Rb* mutant, 38.5%), which we did not see with Sox2 (wild type, 34.8% vs. *Rb* mutant, 37.3%).

**Comparison of Neuronal Populations in Cortical Cultures Lacking Various *Rb* Family Members**

**Neurogenesis still occurs with loss of various *Rb* family members**

To investigate whether loss of *Rb* family members has an effect on the distribution of various cell types in mixed cortical cultures, immunocytochemistry for the various cell type markers was performed on primary cortical neuron cultures lacking various *Rb* family members. To test whether loss of *Rb* family members altered neuronal identity towards a glial cell fate, cultures were analyzed for neuronal markers (Map2a,b, β-tubulin III (TUJ1)), and glial marker (GFAP) on Day 4, as well as the oligodendrocyte marker (O4) on Day 6. There was no significant change in the percentage of cells expressing neuronal markers Map2a,b or β-tubulin III with loss of any individual *Rb* family member or with combined loss of *p107* and *p130* (Figure 4-12 A and B). Additionally, Map2a,b and β-tubulin III staining was morphologically indistinct with loss of various *Rb* family members. Loss of various *Rb* family members also had little effect on the production of astrocytes or oligodendrocytes as marked by GFAP and O4, respectively (data not shown...
Figure 4-12: Primary cortical cultures, in the absence of FGF, maintain neuronal identity with loss of various Rb family members. The frequency of various neuronal markers was monitored in the various Rb family member deficient backgrounds by immunocytochemistry on day 4. There was no significant difference in markers of neuronal identity as assessed by TUJ1(A) and Map2a,b (B) or neuronal maturity as assessed by NeuN(C) in any of the Rb family mutant backgrounds. There was also no change in glial identity as assessed by GFAP(D). The number of embryos analyzed for each marker is shown as n=(n_TUJ1,n_Map2a,b,n_NeuN,n_GFAP).

N= 400-500 cells per experiment
for O4) (Figure 4-12 D). Therefore, within these restricted growing conditions, loss of various Rb family members has little effect on specification of the neuronal lineage in primary cortical cultures. One possibility is that neurons are forming, but they are not becoming fully mature with loss of various Rb family members. Since NeuN is a marker of neurons, the percentage of NeuN+ cells was determined in the various Rb family member deficiencies in Day 4 cultures. However, there was no change detected in the percentage of cells expressing NeuN (Figure 4-12 C), and thus, there was no obvious change in the differentiation of post-mitotic neurons with loss of various Rb family members in cortical neuron culture.

Presence of mature neuronal subtypes in primary cultures lacking various Rb family members

Loss of various Rb family members may not affect the generation of cortical neurons in general, but it may affect the production of certain classes of neurons. This would be consistent with the observation that loss of various Rb family members in vivo does not lead to gross abnormalities in cortical development, but rather results in cell-type specific phenotypes (Ferguson et al., 2005; McClellan et al., 2009). Additionally, interneurons and Cajal-Retzius neurons are located within the subplate and marginal zone of the developing cortex, which is the site that exhibits significant loss of cell death with loss of p107 (Figure 3-11) or p130 (Figure 3-12) or both p107 and p130 (Figure 3-13). To determine if changes in particular neuronal subtypes occurred, immunohistochemical detection of various neuronal markers was used to compare wild type and mutant cortical cultures. The frequency of GABAergic neurons was monitored in Rb family member deficient cortical cultures using the GAD67 marker, and there was no difference in the production of GAD67+ GABAergic neurons with loss of various Rb family members (Figure 4-13 D). Additionally, the frequency of calretinin+ or calbindin+ interneurons with loss of Rb family members was monitored on Day 4, but no significant change in either of these interneuron subtypes was detected (Figure 4-13 B and C).

We investigated if the number of reelin+ cells, an early born subset of calretinin+ cells, changed with loss of Rb family members (Figure 4-13 A). Loss of p107 or p130 alone did not affect the number of reelin+ cells in cortical culture. In contrast, Rb-deficient (NestinCre;RbLoxP/LoxP) cultures showed a trend for decreased abundance in reelin+ cells (wild type = 4.1 ± 0.5%; Rb-deficient = 3.1 ± 0.3%), consistent with previously published in vivo data that showed a decrease in reelin+ Cajal–Retzius neurons in the telencephalic knockout of Rb (Ferguson et al, 2005). Interestingly, there was a statistically
Figure 4-13: Decrease in Reelin+ neurons in \textit{p107-/;p130-/-} cortical culture on Day 4. The frequency different neuronal subtypes was monitored in the various \textit{Rb} family member deficient backgrounds by immunocytochemistry on Day 4. Dual loss of \textit{p107} and \textit{p130} led to a significant decrease in Reelin+ cells in Day 4 cultures (A) (Paired Student’s t-test $p=0.018$). The number of embryos analyzed for each marker is shown as $n=(n_{\text{Reelin}}, n_{\text{Calbindin}}, n_{\text{Calretinin}}, n_{\text{GAD67}})$. N= 400-500 cells per experiment.
significant decrease in reelin+ cells with dual loss of p107 and p130 (p107+/+;p130/- = 3.4 ± 0.2%; p107-/-;p130/- = 2.7 ± 0.2%; Paired Student’s t-test ρ = 0.018). The percentage of reelin+ cells was significantly lower in wild type cultures derived from the p107 heterozygous crosses (129Sv x C57BL/6 background) than wild type cultures derived from the T157 crosses (C57BL/6 background) (Student’s t-test ρ = 0.04), a difference we attributed to variation in the genetic background between experiments. For example, generation of p107-deficient embryos leads to the selection of the 129Sv derived region of chromosome 2, which selects for the 129Sv alleles of the Agouti and E2f1 genes.

Presence of neuronal progenitors in Rb family member deficient cultures

Changes in progenitor populations were monitored in primary cortical cultures deficient in various Rb family members. Nestin is one of the earliest progenitor markers expressed during neuronal development, and no change in its expression was found in primary cortical cultures lacking various Rb family members at Day 4 (Figure 4-14 A). Sox2 is another early neuronal progenitor marker important for the maintenance of the progenitor state, and loss of Rb family members has no effect on the number of Sox2+ cells in Day 4 cultures (Figure 4-14 B).

In contrast to the Sox2 and Nestin results, the percentage of Pax6+ cells did change with loss of Rb family members. Using a polyclonal Pax6 antibody, we saw that loss of Rb led to a significant decrease in the percentage of Pax6+ cells in Day 4 cortical cultures, which was replicated with a monoclonal antibody (Pax6 rabbit polyclonal, Student’s t-test ρ = 0.016; Pax6 monoclonal, Student’s t-test ρ = 0.027) (Figure 4-15 A). This change was specific to loss of Rb, because loss of p107, p130, or dual loss of p107 and p130 did not lead to any change in the frequency Pax6+ progenitors. Within the developing cortex, Pax6+ progenitors give rise to Tbr2+ intermediate progenitors (Englund et al., 2005). To see if the decrease of Pax6+ progenitors was due to faster commitment to the Tbr2+ progenitors in Rb deficient cultures, immunocytochemistry for Tbr2 was performed on cultures lacking various Rb family members on Day 3 (Figure 4-15 B). A modest decrease in the percentage of Tbr2+ cells was observed with loss of Rb (wild type, 45.2 ± 6.8% vs. Rb-deficient, 32.9 ± 1.7%), indicating that the loss of Pax6+ progenitors in Rb-deficient culture was not due to an increase in radial glial cells progressing toward Tbr2+ intermediate progenitors.
Figure 4-14: No changes in the frequency of Nestin or Sox2 progenitors in Rb family deficient cortical cultures on Day 4. The frequency of Sox2 and Nestin positive cells was monitored in the various Rb family member deficient backgrounds by immunocytochemistry on Day 4. There were no significant changes in the number of Sox2+ or Nestin+ cells in any of the Rb family deficiencies tested. Numbers of embryos analyzed per genotype for each marker is shown as n=(n_{Nestin} n_{Sox2}). N= 400-500 cells per experiment.
Figure 4-15: Decrease in the frequency of Pax6+ cortical progenitors with loss of Rb on Day 4. The frequency of Pax6 positive cells was monitored in the various Rb family member deficient backgrounds by immunocytochemistry on Day 4. There was a significant decrease in the frequency of Pax6 progenitors with Rb deficiency (Student's t test: $p = 0.016$) (A). There were no significant changes in the frequency of Tbr2 intermediate progenitors with loss of various Rb family members (B). Numbers of embryos analyzed per genotype for each marker is shown as $n=(n_{Pax6}, n_{Tbr2})$. $N=400-500$ cells per experiment.
Unexpected regulation of the Pax6 progenitor populations by Rb family members

Although Rb-deficient (NestinCre;RbLoxP/LoxP) cultures had fewer Pax6+ progenitors, Pax6+ cells were stronger in fluorescent Pax6 intensity than those in the wild type (RbLoxP/+ or RbLoxP/LoxP) controls (Figure 4-16 B and Appendix Figure A-2). Using the Pax6 monoclonal antibody, we saw a 38% increase in the percentage of the Pax6+ population with intense Pax6 staining with Rb loss (Student’s t-test $p = 0.015$), but we saw no significant change in the intensity of Pax6+ cells with loss of p107 and/or p130. Therefore, we postulated that Rb could be regulating Pax6 expression. Since Rb family members repress E2F target genes, we searched for a possible E2F site within the Pax6 promoter. An E2F site had been reported in the human Pax6 P1 minimal promoter (Zheng et al., 2001), which was conserved within the mouse genome (Figure 4-16 A). The Pax6 gene contains many regulatory elements that control expression in different tissues during development, and the P1 promoter does direct expression within the CNS. Therefore, primers were designed to flank the E2F site, and ChIP was performed with antibodies against Rb family members to see if they occupy the Pax6 promoter. Indeed, moderate binding of Rb family members, particularly p107 and p130, could be seen at the Pax6 promoter in the cortex (Figure 4-16 C). While occupancy by Rb family members may be through the E2F site, Rb family members could be interacting with other transcription factors at neighboring Sp1 or Pax6 sites, since pRB can interact both with Sp1 (Chang et al., 2001) or Pax6 (Cvekl et al., 2004). The potential interaction between Pax6 and Rb at both the transcriptional level as well as the protein level at the Pax6 promoter may be an indication that pRB share a unique genetic relationship. Whether or not levels of Pax6 mRNA increased with loss of Rb family members was not determined.

Identification of the RbP-LacZ Expressing Subpopulations in Primary Cortical Cultures

Regionalization of the Xgal signal in Chapter 2 suggested to us that neuronal populations could be specifically positive or negative for Xgal. The deregulation of RbP-LacZ with conditional loss of Rb or dual loss of p107 and p130 in the developing mouse cortex could be due to changes in frequency or intensity of Xgal staining specifically within these Xgal+ neuronal populations. To explore this possibility, the identity of the RbP-LacZ cell was determined in primary cortical culture, which allowed for single cell analysis using a novel double staining approach (Xgal staining followed by immunohistochemical staining for neuronal markers). This approach was used first on wild type cultures and then followed by
Conservation of E2F site in mouse Pax6 P1 promoter

A

GAGCATCCAATCGGCTGGCGCGAGG

CCCCGGCGCTGCTTTGCA

TAAAGCAATATTTTGTG

Mouse

Pax6

GAGCATCCAATCGGCTGGCGCGAGG

CCCCGGCGCTGCTTTGCA

TAAAGCAATATTTTGTG

Human

E2F

TGAGGAGCGACGCTGGTGAATGTTTCTGAGGAT

TGAGGAGCGACGCTGGTGAATGTTTCTGAGGAT

GATGACAGAAGCGCTTTCTGAGGAT

Sp1

TGAGGAGCGACGCTGGTGAATGTTTCTGAGGAT

TGAGGAGCGACGCTGGTGAATGTTTCTGAGGAT

GATGACAGAAGCGCTTTCTGAGGAT

Sp1

B

Intensity

% Pax6+ High

Day 4

C

Figure 4-16: Rb family members may regulate the Pax6 P1 promoter in the E14.5 cortex.

The Pax6 P1 promoter, which directs expression to the CNS and lens, contains an E2F site, which is conserved between mouse and humans (A). Immunocytochemistry using a monoclonal antibody to Pax6 showed that the intensity of Pax6+ staining (Pax6+ High/Total Pax6+) increased significantly with Rb deficiency in Day 4 cultures (Student’s t-test p = 0.015), implicating possible transcriptional regulation of Pax6 by Rb family members (B). Primers were developed that flanked the E2F site of the Pax6 promoter and ChIP was performed on E14.5 C57BL/6 cortex using antibodies to the various pocket proteins (C). Moderate binding of Rb family members was seen at the Pax6 P1 promoter in the E14.5 cortex.
examination of the identity of the *RbP-LacZ* expressing cells in primary cultures deficient in various *Rb* family members.

Detection of neuronal maturity and *LacZ* activity through co-staining

Primary cortical cultures were stained for 4 hours in X-gal solution on Day 6 to obtain an intense Xgal signal without losing cell integrity. This Xgal staining modification allowed the subsequent immunocytochemical detection of the various cell type markers. The majority of cells in our wild type primary cortical culture system showed a neuronal phenotype as seen by immunostaining for β-tubulin III and Map2a,b. To detect double positive cells, Xgal+ cells were identified first and then scored by immunofluorescence to see if they also expressed each neuronal marker tested. Indeed, 100% of Xgal+ cells were positive for β-tubulin III and 98.6 ± 1.4% of Xgal+ cells were positive for Map2a,b (Figure 4-17 B, C, and H). To confirm the neuronal identity of the cells, Xgal stained wild type *T157* cultures were tested with GFAP and O4 antibodies, experiments which revealed that only a very small percentage of Xgal+ cells were positive for the glial cell markers; 0.5 ± 0.5% of Xgal+ cells were GFAP+ (Figure 4-17 G and H) and 0.7 ± 0.4% were O4+ (Figure 4-17 F and H). This confirmed that the majority of *RbP-LacZ* cells are of a neuronal lineage in the wild type primary cortical culture.

Previous studies reported that *Rb* expression correlates with differentiation of PC-12 cultured cells into neurons (Gill et al., 1998; Slack et al., 1993), suggesting that *Rb* expression plays an important role in mature neurons. We investigated whether *RbP-LacZ* activity correlated with expression of the *NeuN* marker, which marks mature neurons. Indeed, 65.80 ± 15.8% of Xgal+ cells in wild type *T157* culture were NeuN+ (Figure 4-17 D and H), indicating that many of the *RbP-LacZ* expressing cells were mature neurons. Yet, Xgal+ cells only represented 16.6 ± 1.5% of the NeuN expressing population. Therefore, although many cells with *RbP-LacZ* activity are NeuN+, *RbP-LacZ* activity is not a general marker of mature neurons.

Xgal+ neurons were further double stained for additional markers to determine if they segregate with a certain subtype of mature neuron (Figure 4-18). The GABAergic marker, GAD67, co-stained with 4.4 ± 0.7% of Xgal+ cells, representing only 4.3 ± 1.3% of the GAD67 of the population (Figure 4-18 G and H). calbindin+ cells accounted for 5.7 ± 2.7% of the Xgal+ cells, which represents 14.3 ± 6.9% of the calbindin+ population (Figure 4-18 D and H). Double staining of wild type *T157* cultures for Xgal and
<table>
<thead>
<tr>
<th>Marker</th>
<th>n</th>
<th>Xgal</th>
<th>Marker</th>
<th>Xgal+ Marker+ x100</th>
<th>Xgal+ Marker+ / Marker+ x100</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUJ1</td>
<td>2</td>
<td>11.3 ± 1.6%</td>
<td>90.8 ± 0.7%</td>
<td>100.0 ± 0.0%</td>
<td>12.6 ± 4.2%</td>
</tr>
<tr>
<td>Map2</td>
<td>3</td>
<td>10.6 ± 1.2%</td>
<td>87.1 ± 1.9%</td>
<td>98.6 ± 1.4%</td>
<td>12.3 ± 3.0%</td>
</tr>
<tr>
<td>NeuN*</td>
<td>2</td>
<td>8.1 ± 0.5%</td>
<td>33.5 ± 2.9%</td>
<td>65.8 ± 15.8%</td>
<td>16.6 ± 1.5%</td>
</tr>
<tr>
<td>O4</td>
<td>3</td>
<td>10.1 ± 0.3%</td>
<td>2.6 ± 0.2%</td>
<td>0.7 ± 0.4%</td>
<td>2.5 ± 1.3%</td>
</tr>
<tr>
<td>GFAP</td>
<td>3</td>
<td>8.5 ± 0.9%</td>
<td>3.0 ± 0.8%</td>
<td>0.5 ± 0.5%</td>
<td>0.7 ± 0.7%</td>
</tr>
</tbody>
</table>
Figure 4-17: A subset of mature neurons shows RbP-LacZ activity in primary cortical cultures on Day 6. Cortical neurons from wild type T157 cultures on Day 6 were stained with Xgal for four hours and subsequently processed for immunocytochemistry for neuronal and glial markers. DAPI and AlexaFluor555 images are overlaid and are accompanied by light images of the same fields. Arrows show the same cells by either immunofluorescence or light imaging. Xgal+ cells are positive for neuronal markers TUJ1 (B), Map2a,b (C) and NeuN (D) but not glial markers O4 (F) and GFAP (G). Panel H summarizes the counts for each marker. n= number of embryos analyzed; N= 300 – 600 DAPI cells analyzed.
### Table:

<table>
<thead>
<tr>
<th>Marker</th>
<th>n</th>
<th>Xgal</th>
<th>Marker</th>
<th>Xgal⁺ Marker⁺ x100</th>
<th>Xgal⁺ Marker⁺ x100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calretinin</td>
<td>3</td>
<td>10.8 ± 1.2%</td>
<td>6.0 ± 0.9%</td>
<td>16.3 ± 2.6%</td>
<td>28.5 ± 3.0%</td>
</tr>
<tr>
<td>Calbindin</td>
<td>3</td>
<td>13.0 ± 1.0%</td>
<td>5.3 ± 0.6%</td>
<td>5.7 ± 2.7%</td>
<td>14.3 ± 6.9%</td>
</tr>
<tr>
<td>Reelin</td>
<td>3</td>
<td>12.1 ± 1.1%</td>
<td>6.5 ± 0.9%</td>
<td>12.0 ± 0.2%</td>
<td>22.7 ± 2.0%</td>
</tr>
<tr>
<td>GAD67</td>
<td>3</td>
<td>9.7 ± 1.2%</td>
<td>10.4 ± 0.8%</td>
<td>4.4 ± 0.7%</td>
<td>4.3 ± 1.3%</td>
</tr>
</tbody>
</table>
Figure 4-18: A fraction of interneurons and Cajal-Retzius neurons show RbP-LacZ activity. Cortical neurons from wild type T157 cultures on Day 6 were stained with Xgal for four hours, and then subsequently processed for immunocytochemistry for markers of various mature neuron subtypes. A fraction of Cajal-Retzius neurons, marked by Calretinin (B) and Reelin (F) expression, are Xgal+ in Day 6 culture. A smaller percentage of GABAergic (G) and Calbindin (D) interneurons show weak RbP-LacZ activity. Panel H summarizes the counts for each marker. n= number of embryos analyzed; N= 300 - 600 DAPI cells analyzed.
calretinin revealed that 16.3 ± 2.6% of the Xgal+ cells were also calretinin+, and conversely, Xgal+ cells accounted for 28.5 ± 3.0% of the calretinin+ population (Figure 4-18 B and H). To test whether these Xgal+ calretinin+ cells represented part of the Cajal–Retzius neuron population, double staining for Xgal and reelin was performed, which showed that 12.0 ± 0.2% of Xgal+ cells were also reelin+, representing 22.7 ± 2.0% of the reelin+ population (Figure 4-18 F). Thus, a subset of RbP-LacZ activity identifies a subset of Cajal–Retzius neurons in wild type T157 primary cortical cultures.

**Detection of neuronal progenitors and LacZ activity through co-staining**

Progenitor populations represent a large component of primary cortical neuron cultures, and so the majority of Xgal+ cells not identified using mature neuron markers could belong to progenitor populations. Double staining for Xgal and progenitor markers was performed on wild type T157 cultures to investigate whether RbP-LacZ activity identified any progenitor populations (Figure 4-19). The early neural progenitor marker, Sox2, did not correlate with Xgal positivity; 0.5 ± 0.5% of Xgal+ cells expressed Sox2, reflecting 0.2 ± 0.2% of the Sox2 population (Figure 4-19 C and F). Nestin also marks some of the earliest neuronal progenitors but does not appear to correlate with RbP-LacZ activity, because only 3.2 ± 1.6% of Xgal+ cells also were Nestin+, representing only 1.4 ± 0.7% of the Nestin+ population (Figure 4-19 E and F). Therefore, RbP-LacZ activity does not correspond to these markers of the earliest neuronal progenitors. To test if RbP-LacZ activity identifies Pax6+ progenitors, we performed double staining for Xgal and Pax6. Importantly, we determined that 66.8 ± 9.4% of Xgal+ cells were also positive for Pax6 (Figure 4-19 B and F), indicating that the majority of cells with RbP-LacZ activity are part of the Pax6 progenitor population. Interestingly, 65.7± 4.7% of the Pax6+ population were also Xgal+, indicating that the majority of Pax6+ progenitors in Day 6 primary cortical neuron cultures show RbP-LacZ activity. Additionally, we observed that the majority of double positive cells that stained a dark blue after only four hours of Xgal treatment expressed nearly always expressed Pax6, indicating that high Rb promoter activity is strongly coupled to Pax6 expression, a result that will be pursued in later sections.

The distribution of RbP-LacZ expressing cells amongst the different cortical neuron populations is summarized in Figure 4-20. The majority of RbP-LacZ expressing neurons (TUJ1+, Map2a,b+) belong to a Pax6+ lineage and at least some of these have achieved a NeuN+ status(Figure 4-20 A). With the exception of the Pax6+ progenitor pool, the double positive (Xgal+ Marker+) neurons represent only a
Figure 4-19: Pax6 progenitors represent the majority of Xgal+ cells in Day 6 cortical culture. Cortical neurons from wild type T157 cultures on Day 6 were stained with Xgal for four hours and then subsequently processed for immunocytochemistry for progenitor markers. Very few Xgal+ cells expressed progenitor markers Sox2 (C) or Nestin (E). The majority of Xgal+ cells expressed Pax6 (B), and the strongest Xgal+ cells expressed very high levels of Pax6. Panel F summarizes data from experiments. n= number of embryos analyzed; N= 400-600 DAPI cells analyzed.
Figure 4-20: 

RbP-LacZ expressing cells represent a subset of mature neurons but the majority are Pax6+ neuronal progenitors.

When the distribution of different cell populations is monitored within the Xgal+ population (A), it can be seen that all Xgal+ cells express markers of neuronal identity (TUJ1 and Map2a,b), and a large majority express Pax6 and/or NeuN. When the percentage of Xgal+ cells is monitored within each cell population (B), it is apparent that Xgal+ cells are only a small percentage of the total neuronal population, but represent the majority of the Pax6+ population. n= 3 embryos; N=300-1000 DAPI cells per marker.
small percentage of the total neurons found of that subtype (Marker+) (Figure 4-20 B). \textit{RbP-LacZ} activity was also found within ~25% of both reelin+ and calretinin+ populations, indicating that \textit{Rb} expression is important for the development of Cajal-Retzius neurons. Thus, \textit{RbP-LacZ} activity identifies predominantly Pax6+ progenitor cells in E14.5 cortical neuron cultures, with a smaller percentage corresponding to mature interneurons.

**Changes in \textit{RbP-LacZ} Activity in Neuronal Subpopulations with Loss of \textit{Rb} Family Members**

\textit{RbP-LacZ} activity in neuronal subpopulations with loss of \textit{p107} or \textit{p130}

To determine whether the neuronal populations positive for \textit{RbP-LacZ} activity changed with loss of \textit{Rb} family members, Day 6 \textit{T157} wild type and mutant primary cortical cultures (either deficient in \textit{p107} or \textit{p130} or \textit{Rb} or doubly deficient in \textit{p107} and \textit{p130}) were double stained by first incubating for a short time with Xgal solution, followed by immunocytochemistry with antibodies to various neuronal markers. When comparing \textit{T157;p107+/+} to \textit{T157;p107-/-} cultures, we observed no major differences in the percentage of Xgal+ cells within the different neuronal populations (Figure 4-21 A), with the majority of Xgal+ cells maintaining a Pax6+ and neuronal identity. When comparing \textit{T157;p130+/+} and \textit{T157;p130-/-} cultures, there were no large changes in Xgal+ frequency within the cell types, which constitute the bulk of \textit{RbP-LacZ} activity (Pax6, NeuN, Map2, TUJ1) (Figure 4-21 B). There was a decrease in the percentage of Xgal+calretinin+ cells in the calretinin+ subset of interneurons with \textit{p130} loss, but unfortunately, subsequent dissections did not provide embryos of the proper genotypes for comparison to test if this effect was statistically significant.

\textit{RbP-LacZ} activity in neuronal subpopulations with dual loss of \textit{p107} and \textit{p130}

**Effect of dual loss of \textit{p107} and \textit{p130} on the frequency of \textit{RbP-LacZ} expressing Pax6+ cells**

Dual loss of \textit{p107} and \textit{p130} resulted in only one significant change in the distribution of Xgal+ cells within the different neuronal population tested using our double staining approach. Strikingly, there was a significant decrease in the frequency of the Xgal+Pax6+ population with dual loss of \textit{p107} and \textit{p130} in four separate experiments. (\textit{T157;p107+/+;p130-/-}, 74.5 \pm 5.8\% vs. \textit{T157;p107-/-;p130-/-}, 59.4 \pm 9.4\%; paired Student’s t-test \(p=0.014\)) (Figure 4-21 C).

Since Pax6+ cells give rise to Tbr2+ cells in vivo (Englund et al., 2005), we examined if Tbr2+ cells were also Xgal+, and whether the percentage of Xgal+Tbr2+ cells in the Tbr2+ population changes
Figure 4-21: The frequency of Xgal+Pax6+ cells decreases with dual loss of p107 and p130.

The percentage of Xgal+ cells within the different neuronal subpopulations for the various Rb family deficiencies on Day 6 is summarized. The x-axis is the percentage of Xgal+Marker+ cells within each population tested. There was a significant decrease in the number of Xgal+Pax6+ cells within the Pax6 population with dual loss of p107 and p130 (C) (n= 4 embryos; paired Student's t-test $p= 0.014$). There was a small but significant increase in the number of Xgal+Sox2+ and Xgal+Nestin+ cells with Rb deficiency (n=2 embryos; Student's t-test Xgal+Sox2+: $p= 0.0043$; Xgal+Nestin+: $p= 0.033$). The number of embryos per genotype (n) differed for different markers. N = 250-500 DAPI cells analyzed per marker.
with dual loss of \textit{p107} and \textit{p130}. In fact, a substantial percentage of Xgal+ cells in \textit{T157;p107+/+;p130/-} cultures were Tbr2+ (68.3 ± 10.0%). We found a decrease in the percentage of Xgal+ Tbr2+ cells in the Tbr2+ population with dual loss of \textit{p107} and \textit{p130}, but it was not significant (Figure 4-21 C).

Therefore, we found that in primary cortical cultures, the frequency of Xgal+Pax6+ cells is decreased within the Pax6+ progenitor populations with dual loss of \textit{p107} and \textit{p130}, and since Pax6+ progenitor populations are the majority of Xgal+ cells, the loss of Xgal+Pax6+ cells in the Pax6+ progenitor pool likely contributes to the observed decrease in \textit{RbP-LacZ} activity in the intact embryonic cortex lacking both \textit{p107} and \textit{p130} at E14.5 (Figure 2-3).

\textit{Effect of dual loss of \textit{p107} and \textit{p130} on the intensity of \textit{RbP-LacZ} in Pax6+ cells}

When total cortical population (DAPI+) was analyzed, there was no significant change in the intensity (blueness) of Xgal staining observed with loss of \textit{p107} and \textit{p130} (Figure 4-9). However, it could be that no effect was found in this analysis because it included a mixed population of cells, whereas the analyses of double stained cells suggest that \textit{RbP-LacZ} activity is reduced by loss of \textit{p107} and \textit{p130} in only a specific population of cells. Since the majority of Pax6+ cells display \textit{RbP-LacZ} activity, we asked if there was a detectable decrease in \textit{RbP-LacZ} activity within the Pax6+ subpopulation on a cell-by-cell basis, as judged by the intensity of Xgal staining. To do this, we used saturation level of the Xgal signal as a measure of the output of \textit{RbP-LacZ} activity. Each Xgal+Pax6+ cell was examined using ImageJ to measure the blue saturation of each cell using RGB levels (as described in the Methods Section). Strikingly, the mean blue saturation of Xgal+Pax6+ cells in \textit{T157;p107/-;p130/-} culture was significantly lower than that in \textit{T157;p107+/+;p130/-} culture (Figure 4-22 A), demonstrating that the \textit{RbP-LacZ} reporter is less active with loss of both \textit{p107} and \textit{p130}. There was no difference seen in the blue saturation of Xgal+Pax6+ cells in \textit{Rb}-deficient cultures (Figure 4-22 B). Thus, dual loss of \textit{p107} and \textit{p130} leads to both a decrease in the \textit{frequency} of Xgal+Pax6+ cells in the Pax6+ progenitor population (Figure 4-21), and a decrease in the \textit{activity} of the \textit{RbP-LacZ} reporter within the Pax6+ population (Figure 4-22), supporting a cell-autonomous mechanism for the decrease in \textit{RbP-LacZ} activity seen in the \textit{T157;p107/-;p130/-} cortex.
Figure 4-22: RbP-LacZ activity is decreased Xgal+Pax6+ cells with loss of p107 and p130

The output of RbP-LacZ activity was determined by measuring the saturation of Xgal staining from light images of Xgal+Pax6+ co-stain experiments. Saturation is calculated using the formula: Saturation=(Blue_{RGB} - Min_{RGB})/Blue_{RGB} using RGB levels determined using the image analysis software, Image J. The level of blue saturation is shifted lower in Xgal+ Pax6+ co-stained cells in T157;p107-/-;p130-/- culture relative to T157;p107+/+;p130-/- culture. There was no shift seen with Rb deficiency (B). Panel C shows that the mean blue saturation of Xgal+Pax6+ cells was significantly lower in T157;p107-/-;p130-/- culture as compared to T157;p107+/+;p130-/- cultures (Student’s t-test p < 0.00001). N= number of Xgal+Pax6+ cells per genotype.
**RbP-LacZ activity in neuronal subpopulations with loss of Rb**

**Effect of Rb loss on RbP-LacZ activity in progenitor populations**

Loss of Rb showed no significant differences in the percentage of Xgal+ cells within the different mature neuron populations or within the Pax6+ progenitor populations (Figure 4-21 D). Loss of Rb did lead to a significant increase in RbP-LacZ activity within the early (Sox2+ or Nestin+) progenitor populations (Figure 4-21 D). While other wild type and mutant cultures tested had only the occasional Xgal+ Sox2+ cell, Rb-deficient (T157;NestinCre;RbLoxP/LoxP) cultures showed a significant increase in the Xgal+ Sox2+ cells in the Xgal+ population (6.7 ± 0.7%), relative to that found in wild type (T157;RbLoxP/+ or T157;RbLoxP/LoxP) cultures (0.6 ± 0.6%) (Student’s t-test $\rho = 0.020$). There was also a significant increase in the percentage of Xgal+ Sox2+ cells in the Sox2+ (T157;RbLoxP/LoxP, 0.9 ± 0.5% vs. T157;NestinCre;RbLoxP/LoxP, 5.5 ± 0.6%; Student’s t-test $\rho = 0.020$) (Figure 4-21 D).

Additionally, there was an increase in the Xgal+ Nestin+ population with loss of Rb. The percentage of Xgal+ cells that were Xgal+ Nestin+ increased from 1.0 ± 1.0% to 5.7 ± 0.4% with Rb loss, and the percentage of Nestin+ cells that were Xgal+ Nestin+ increased from 0.8 ± 0.8% to 5.6 ± 0.4% with Rb loss (Student’s t-test $\rho = 0.0043$ and $\rho = 0.033$, respectively). Thus, a small percentage of early neuronal progenitors express RbP-LacZ in Rb-deficient culture. Although this increase in RbP-LacZ activity in Sox2+ and/or Nestin+ involves a small fraction of the total population of Xgal+ cells, over time this progenitor population could expand substantially, and could contribute to increased RbP-LacZ activity in the cortex of Rb-deficient embryos. Whether this is the case or not would require further study.

**Changes in nuclear size with Rb loss in primary cortical culture**

How could Rb-deficient embryos display increased RbP-LacZ activity (Figures 2-8 and 2-13), but isolated neurons from Rb-deficient embryos show no increased Xgal+ frequency or intensity (Figures 4-9 and 4-22)? We noticed that the nucleus size of RbP-LacZ cells appeared much larger in Rb-deficient cultures than in the wild type cultures, which was more obvious when these cells were Xgal+. Therefore, we measured the cross-sectional nuclear area of RbP-LacZ expressing cells of the different co-stained populations in wild type and Rb-deficient primary cortical cultures. In Day 6 cultures, there was a pronounced increase in the cross-sectional area of nuclei of Xgal+ Pax6+ cells in Rb deficient nuclei (Figure 4-23 B and D). Interestingly, the increase in nuclear area was not specific to the Xgal+ Pax6+
Figure 4-23: Loss of Rb leads to an increase in nuclear size in cortical culture on Day 6. The cross-sectional area of nuclei were measured using DAPI images from co-stain experiments using Image J. There is a shift to larger nuclei in Rb-deficient culture whether counting all cells from a mIgG (A) stained culture, or if one counts within the Xgal+Pax6+ (B) or Xgal+NeuN+ (C) populations. There was a significant increase in nuclear area in Rb deficient culture regardless of Xgal status and in most populations assessed (D).

<table>
<thead>
<tr>
<th></th>
<th>Average Area of Xgal+ Nucleus (pixels )</th>
<th>T157; RbLoxP/LoxP</th>
<th>T157; NestinCre;RbLoxP/LoxP</th>
<th>Ratio (Mutant/WT)</th>
<th>Student’s t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>mIgG</td>
<td>4012 ± 100 (N=104)</td>
<td>5450 ± 195 (N=100)</td>
<td>1.36</td>
<td>p&lt; 0.000001</td>
<td></td>
</tr>
<tr>
<td>Xgal+Pax6+</td>
<td>6076 ± 140 (N=85)</td>
<td>7324 ± 255 (N=101)</td>
<td>1.21</td>
<td>p = 0.00021</td>
<td></td>
</tr>
<tr>
<td>Xgal+NeuN+</td>
<td>4853 ± 124 (N=49)</td>
<td>564 ± 2282 (N=45)</td>
<td>1.16</td>
<td>p = 0.009</td>
<td></td>
</tr>
<tr>
<td>Xgal+Calretinin+</td>
<td>5323 ± 388 (N=11)</td>
<td>7047 ± 747 (N=14)</td>
<td>1.32</td>
<td>p = 0.08</td>
<td></td>
</tr>
<tr>
<td>Xgal+Reelin+</td>
<td>5609 ± 383 (N=11)</td>
<td>6561 ± 528 (N=16)</td>
<td>1.17</td>
<td>p = 0.20</td>
<td></td>
</tr>
</tbody>
</table>
**A**

![Graphs showing area of nucleus](image)

**B**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average Area (pixels)</th>
<th>N</th>
<th>Ratio (Mutant/WT)</th>
<th>Student’s t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RbLoxP/LoxP</em></td>
<td>4680 ± 95.8</td>
<td>204</td>
<td>1.10</td>
<td>p= 0.0005</td>
</tr>
<tr>
<td><em>NestinCre;RbLoxP/LoxP</em></td>
<td>5154 ± 96.0</td>
<td>212</td>
<td>1.10</td>
<td></td>
</tr>
<tr>
<td><em>p107+/++;p130/-</em></td>
<td>5146 ± 88.6</td>
<td>203</td>
<td>0.97</td>
<td>N.S.</td>
</tr>
<tr>
<td><em>p107-/-;p130/-</em></td>
<td>4988 ± 96.9</td>
<td>209</td>
<td>0.97</td>
<td>N.S.</td>
</tr>
<tr>
<td><em>p107+/+</em></td>
<td>4670 ± 101.8</td>
<td>192</td>
<td>0.98</td>
<td>N.S.</td>
</tr>
<tr>
<td><em>p107-/-</em></td>
<td>4574 ± 92.0</td>
<td>192</td>
<td>0.98</td>
<td>N.S.</td>
</tr>
<tr>
<td><em>p130+/+</em></td>
<td>4429 ± 105.5</td>
<td>190</td>
<td>1.01</td>
<td>N.S.</td>
</tr>
<tr>
<td><em>p130-/-</em></td>
<td>4482 ± 92.2</td>
<td>195</td>
<td>1.01</td>
<td></td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>Marker</th>
<th>Average Nucleus Area (Pixels)</th>
<th>Ratio (Mutant/WT)</th>
<th>Student’s t-test (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>4680 ± 95.8</td>
<td>1.10</td>
<td>0.0005</td>
</tr>
<tr>
<td>Sox2</td>
<td>4334 ± 103.0</td>
<td>1.10</td>
<td>0.004</td>
</tr>
<tr>
<td>Nestin</td>
<td>3922 ± 93.1</td>
<td>1.19</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Pax6</td>
<td>4278 ± 112.2</td>
<td>1.16</td>
<td>0.0001</td>
</tr>
<tr>
<td>NeuN</td>
<td>4657 ± 87.8</td>
<td>1.06</td>
<td>0.04</td>
</tr>
<tr>
<td>Reelin</td>
<td>3497 ± 111.8</td>
<td>1.13</td>
<td>0.02</td>
</tr>
<tr>
<td>Gad67</td>
<td>3912 ± 105.6</td>
<td>1.20</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Calretinin</td>
<td>5229 ± 143.8</td>
<td>0.97</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
**Figure 4-24: An increase in nuclear size is specific to Rb deficiency in Day 4 culture.**

The cross-sectional area of nuclei were measured using DAPI images from Day 4 antibody-only immunocytochemistry experiments in the various Rb family member deficiencies. There is an increase in nuclear size with loss of Rb (A) that is statistically significant (B), but nuclear size does not significantly differ with loss of p107 (B), p130 (B), or dual loss of p107 and p130 (A, B). The increase in nuclear size with Rb deficiency does not appear to be specific to certain neuronal subtypes, but can be seen both within neuronal progenitors as well as mature neurons (C). (Number of cells analyzed for each marker = 120 - 200). N= number of DAPI cells analyzed.
population, but could be seen in the other Xgal+ populations (e.g. Xgal+NeuN+, Figure 4-23 C and D and Xgal+calretinin+, Figure 4-23 D) as well as in unstained cells (mIgG, Figure 4-23 A and D). The increased nuclear area with loss of Rb could contribute to a more visible region of RbP-LacZ activity (Xgal+) within the cortex, because a modest 21% increase in nuclear cross-sectional area in Pax6+Xgal+ translates to a 30% increase in nuclear volume. Since the LacZ reporter encodes a NLS, the Xgal signal is concentrated in the nucleus of RbP-LacZ expressing cells, giving rise to larger blue nuclei in the Rb-deficient cortex. These larger blue nuclei could account for the apparent increase in RbP-LacZ activity within the cortex. Thus, a cell autonomous increase in nuclear size in the Rb deficient neurons could lead to an apparent increase in RbP-LacZ activity within Rb-deficient embryos.

To ensure that the increase in nuclear area with loss of Rb was not a result of the double staining process, we performed nuclear area measurements using DAPI stained cells from Day 4 single staining experiments. We again found a highly significant increase in cross-sectional nuclear area with loss of Rb in Day 4 cultures (Figure 4-24). This increase was specific to loss of Rb, because we did not see any changes in nuclear area with loss of any other Rb family member (Figure 4-24 B); however, this increase was not specific to any particular cell type (Figure 4-24 C). The magnitude of the increase observed in single stained cells on Day 4 is less than the increase seen in double stained cells on Day 6, which in part is due to the relative state of maturity of the primary culture. Regardless, Rb-deficient cultures clearly are composed of neurons with greater nuclear size and thus, the increased nuclear size requires increased RbP-LacZ activity to give the appearance that the Xgal intensity remains unchanged. We believe that the increased nuclear area of the Rb-deficient cells in cortical culture may be the result of increased ploidy that occurs specifically with loss of Rb, which will be further discussed in the Discussion.

**Summary**

While the loss of Rb family members only had small effects in mixed cortical culture as a whole, there were larger changes in neuronal progenitors and proliferation with loss of Rb that were not seen with loss of p107 and/or p130. Double staining of Xgal+ cells with neuronal markers revealed that loss of Rb and dual loss of p107 and p130 have different effects in primary cortical neuronal culture, which contribute to the differences seen in Xgal staining within intact mutant embryos. The loss of Rb does not appear to change the expression of RbP-LacZ, but it does change the size of the cell nucleus, which
likely contributes to the apparent increase of $RbP$-$LacZ$ activity in vivo in a cell autonomous manner (Figure 4-25). Since larger nuclei would require greater $RbP$-$LacZ$ expression to produce an equivalent level of Xgal saturation, there is likely an increase in $RbP$-$LacZ$ activity within $Rb$-deficient neurons, which we were unable to measure. Dual loss of $p107$ and $p130$ leads to a decrease in $RbP$-$LacZ$ activity within the Pax6+ population, both in frequency and intensity of $RbP$-$LacZ$ activity as determined by changes in Xgal staining (Figure 4-26). Since Pax6+ progenitors are the predominant cell type expressing the $RbP$-$LacZ$ reporter, the changes in $RbP$-$LacZ$ expression within Pax6 progenitors represent a cell-autonomous mechanism by which loss of $p107$ and $p130$ leads to decreased $RbP$-$LacZ$ expression in the developing cortex.
Figure 4-25: Loss of *Rb* can increase *RbP-LacZ* activity by both cell-autonomous and non-cell autonomous mechanisms. The loss of *Rb* increases neurogenesis within the developing cortex (Chapter 3). Increased numbers of cells would entail the existence of more cells with *RbP-LacZ* activity, even if the proportion of such cells in the population is constant. The greater number of cells with *LacZ* activity would visually appear as increased Xgal staining at the level of the cortex. This would constitute a non-cell autonomous mechanism. However, loss of *Rb* also increases nuclear size (Chapter 4). This non-cell autonomous change in nuclear size could be an indicator of a cell autonomous increase in *LacZ* activity. In order for a nucleus of a larger size to have the same intensity of Xgal staining as a smaller nucleus, the larger nucleus would require a higher level of *LacZ* activity. Thus, this cell-autonomous mechanism could explain why Xgal staining intensity for wild type and *Rb*-deficient mice is equivalent at the level of individual cells, but is more intense for *Rb*-deficient mice at the level of the cortex.
Figure 4-26: p107 and p130 function as activators of RbP-LacZ expression in a cell autonomous manner. A decrease both in the frequency of Xgal+Pax6+ progenitors, as well as the intensity of the Xgal signal within Pax6+ progenitors, in primary neuronal culture with dual loss of p107 and p130, may explain the decrease in RbP-LacZ expression seen within the developing cortex.
Chapter 5: DISCUSSION

Rb Family Members Regulate the Rb Promoter In Vivo

pRB acts directly and indirectly to repress Rb expression in the developing cortex

Our initial hypothesis was that Rb family members act as repressors of the Rb promoter in the nervous system, potentially through E2F regulation. Since increased Rb expression is associated with increased differentiation in other systems (Coppola et al., 1990; Slack et al., 1993; Kiess et al., 1995; Gill et al., 1998), we postulated that regulation of Rb expression by Rb family members might be a way that cell cycle control and differentiation were linked. However, what we found instead is much more complex. Whole mount Xgal staining of Rb-deficient embryos and cortices constantly displayed an increase in RbP-LacZ activity relative to that seen in wild type embryos, consistent with pRB acting as a repressor of its own promoter. Yet, when we sought changes in RbP-LacZ activity on a cell-by-cell basis in primary cortical neuron culture, we did not see any increase in RbP-LacZ activity, whether we measured the frequency or the intensity of Xgal staining within primary cortical cultures. This suggests that it is the secondary effects of Rb loss, such as the increases in proliferation and increases in nuclear size, that are predominantly responsible for the increase in RbP-LacZ activity seen within the embryo. An increase in proliferation with Rb loss could easily increase the absolute number of RbP-LacZ cells leading to the appearance of more RbP-LacZ activity, and this idea is supported with our finding that Rb-deficient embryos have consistently larger cortices. Similarly, the general increase in nuclear area of Rb-deficient cells leads to an increased size and visibility of Xgal+ cells that, by eye, would appear as increased RbP-LacZ activity in the cortex.

Yet, the increase in nuclear area with Rb deficiency complicates the analysis of RbP-LacZ activity. Increased LacZ activity could be masked by the increased size of the Rb-deficient nucleus because a larger nucleus would require more RbP-LacZ activity to produce the same Xgal signal as a smaller nucleus. Therefore, it may be that RbP-LacZ activity is higher with loss of Rb, but this effect is obscured by the secondary effect of increased nuclear size. Using spectrophotometric analysis to measure RbP-LacZ activity within the Xgal+ subpopulations could potentially answer this question in a more quantitative manner. However, since only a small percentage of cells show RbP-LacZ activity, it
would be difficult to see any difference unless a way of isolating the \( RbP-LacZ \) active cells, while maintaining cell viability, was devised.

Since we found that pRB occupies the \( Rb \) promoter in the cortex by ChIP, it is most likely playing a negative role in \( Rb \) regulation, but perhaps not to the same extent as we had previously thought. The \( RbP(E2FAAA)-LacZ \) reporter mouse, which contains a mutated \( E2F \) site in the critical cassette of the \( Rb \) promoter, exhibited a dramatic increase in \( LacZ \) activity within the nervous system (Agromayor et al., 2006). Yet, we found a more modest increase in \( LacZ \) activity with loss of \( Rb \), indicating that loss of \( Rb \) repression most likely requires the loss of multiple \( Rb \) and/or \( E2F \) family members. This is supported by our ChIP data showing the continued presence of \( E2F4 \) and \( p130 \), both known repressors, at the \( Rb \) promoter in \( Rb \)-deficient cortex. The generation of triple knockout embryos for \( Rb \) family members has been described (Wirt et al., 2010), using conditional alleles for both \( Rb \) and \( p130 \), which would allow for the generation of \( T157 \) embryos deficient in all three \( Rb \) family members, albeit at a low frequency. This may lead to a greater increase in \( RbP-LacZ \) activity within the cortex, but it is unlikely to replicate the \( RbP(E2FAAA)-LacZ \) reporter, which eliminates all \( E2F \) regulation. Regulation of the \( Rb \) promoter by \( Rb \) family independent \( E2Fs \) (i.e. \( E2F6 \), \( E2F7 \), and \( E2F8 \)) is also possible, particularly since we did find \( E2F6 \) at the \( Rb \) promoter in juvenile cortex. Further ChIP experiments would determine whether \( E2F6-8 \) occupy the \( Rb \) promoter in the absence of various \( Rb \) family members.

The one place where we did see a cell-autonomous increase in frequency of \( RbP-LacZ \) activity with \( Rb \) deficiency was in Sox2 and Nestin progenitor populations. Increased proliferation of the Sox2 and/or Nestin progenitors that express \( RbP-LacZ \) would result in the expansion of Xgal+ cells in the \( Rb \)-deficient cortex. While the increases in the percentages of Nestin+ and Sox2+ progenitors with \( RbP-LacZ \) activity were small, they do indicate that the \( Rb \) promoter is being turned on with loss of \( Rb \) in cell types where we rarely saw \( RbP-LacZ \) activity. This indicates that pRB may still act as a direct repressor of \( Rb \) expression, if only in specific cell populations.

\textit{p107 and p130 activate Rb expression in a cell-autonomous manner}

Intriguingly, we have discovered that \( p107 \) and \( p130 \) act as activators of \( Rb \) expression in the developing cortex, which is in contrast to the known roles of \( p107 \) and \( p130 \) as co-repressors of \( E2F \) dependent transcription. Whole mount staining of embryos and cortices consistently showed a decrease
in RbP-LacZ activity with dual loss of p107 and p130. When we examined isolated cells in primary cortical culture, we discovered that there was a decrease in both the intensity and frequency of cells with RbP-LacZ activity with dual loss of p107 and p130, but that the decrease was restricted to Pax6+ progenitors. This restriction to a small Pax6 population within p107-/-;p130-/- primary cortical cultures, may explain why there was no decrease in Rb levels by real time RT-PCR in the doubly deficient cortex.

While there is ample evidence that Rb can interact with other transcription factors and promote the activation of genes needed for differentiation, thus far, there is little data that p107 and/or p130 can act as activators of gene expression. We found that both p107 and p130 occupy the Rb promoter, suggesting that they directly act to regulate Rb expression. However, it is more likely that secondary effects of p107 and p130 loss are affecting Rb expression levels, than it is that p107 and p130 are direct activators of the Rb promoter. Rossi et al. (2002) found that loss of p107 leads to decreased expression of Cbfa1, an osteogenic factor required for differentiation of chondrocytes, but they speculated that this is due to defect in cell cycle exit, and is not a direct effect of p107 loss on Cbfa1 transcription. Alternatively, it is possible that there is increased occupancy by pRB at the Rb promoter in the p107-/-;p130-/- cortex that we did not find in the limited ChIP experiments performed. While loss of p107 and p130 did not show major de-regulation of Rb family independent E2Fs at E12.5, it is possible that E2F6, E2F7, and/or E2F8 show increased binding to the Rb promoter, resulting in its repression. In a similar manner, we did not determine if the presence of activators, such as ATF or Sp1, at the Rb promoter was affected by loss of Rb family members. Loss of these activators at the Rb promoter easily could lead to less RbP-LacZ activity within the cortex.

Alternatively, a decrease in RbP-LacZ activity in p107-/-;p130-/- cortex could be due to epigenetic changes at the Rb promoter. The region surrounding the critical cassette of the human RB and mouse Rb promoter is very GC rich, and silencing of the RB promoter by methylation of CpG islands is one mechanism that leads to silencing without mutation at the RB locus in retinoblastoma (Sakai et al., 1991b; Ohtani-Fujita et al., 1997; Mancini et al., 1997). Therefore, p107;p130 deficiency could indirectly lead to increased DNA methylation at the Rb promoter. One possible mechanism could be through the upregulation of the DNA methyltransferase, Dnmt-1, which contains an E2F site in its promoter, and whose expression increases with loss of Rb in prostate epithelial cells (McCabe et al., 2005). A similar
deregulation of Dnmt-1 in the p107-/-;p130-/- cortex could lead to hypermethylation of the Rb promoter and a decrease in Rb expression in Pax6 progenitors, although we might expect similar deregulation of Dnmt-1 with Rb loss. This could be explained by differential regulation of E2F targets in the cortex by Rb, p107, and p130. For example, we found that deregulation of E2f1 occurs with loss of Rb, but not with loss of p107 and/or p130.

Similar to pRB, both p107 and p130 can recruit histone deacetylase, HDAC1, and the histone methyltransferase SUV39H1, which lead to repressive chromatin marks and silencing of gene expression (Rayman et al., 2002; Macaluso et al., 2003; Chinnam and Goodrich, 2011). Knowing this, loss of p107 and p130 should lead to an increase in active histone marks at the Rb promoter and increased RbP-LacZ activity. Instead, with loss of p107 and p130, we see a decrease or no change in active histone marks by ChIP at the Rb promoter, which is consistent with a decrease in RbP-LacZ activity, but not with the known interactions of p107 and p130 with histone modifiers. A more comprehensive look at specific acetylation and methylation marks at the Rb promoter in wild type and p107- and p130- deficient cortex could uncover the possible mechanism. For example, we do not know the status of trimethylation at histone H3 at lysine 27 in p107-/-;p130-/- cortex. This is a repressive histone mark instituted by the polycomb repressive complex 2 (PRC2), and is found at genes needed for differentiation in an Rb-dependent and -independent manner (Blais et al., 2007). Having a larger picture of the multitude of histone marks at the Rb promoter in p107-/-;p130-/- cortex could delineate a mechanism of how p107 and p130 act as activators of Rb expression.

Rb Family Members and Pax6 Share a Close Relationship In Neuronal Development

Our work has discovered multiple new connections between Pax6 and Rb family members, suggesting that these regulators share a close relationship during neuronal development. Previous studies have highlighted the role of each of these regulators in neurogenesis, but before this study, none has indicated a critical relationship between Pax6 and Rb in neurogenesis, and there has been no link made before between Pax6 and either p107 or p130. We have found that (1) Rb promoter activity increases specifically in Pax6+ progenitors, (2) Pax6 levels (protein) increases with Rb loss, (3) dual loss of p107 and p130 decreases the frequency of Xgal+Pax6+ cells in the Pax6+ progenitor pool and the
strength of $Rb$ promoter activity in these progenitors and finally, (4) $Rb$ family members are bound to the Pax6 P1 promoter.

Pax6 belongs to a family of transcription factors, which share a paired-box DNA binding domain and a homeodomain, and regulates both progenitor cell self-renewal and neuronal differentiation and patterning in a context-dependent manner (Robson et al., 2006). Pax6 expression occurs in the central and peripheral nervous system, the eye (retina and lens) and the pancreas, the first two of which are key sites for $Rb$ expression. Loss of Pax6 function in the Sey/Sey mouse leads to aberrations in the development of the retina, lens, and cortex and to perinatal lethality (Hill et al., 1991). While loss of Pax6 and loss of $Rb$ are not equivalent events, there are similarities in tissues affected and types of abnormalities observed (Figure 5-1). Although Pax6 is required for the formation of the lens placode in early eye development, both Pax6 and $Rb$ are required for proper lens differentiation. Loss of Pax6 leads to smaller cortex due to early cell cycle exit and premature differentiation of progenitor cells (Quinn et al., 2007), which complements the $Rb$ mutant phenotype of larger cortex (MacPherson et al., 2003; Ferguson et al., 2002). Additionally, Pax6 and $Rb$ are both important for proper retinal development. Loss of Pax6 in retinal progenitor cells leads to a decrease in proliferation and the inability of cells to differentiate into the multiple cell types of the retina; only amacrine interneurons can develop (Phillips et al., 2005; Marquardt et al., 2001). Retinal development is impaired in chimeric Sey/Sey mice (Collinson et al., Dev Biol 2003) and PAX6 mutations in humans leads to aniridia and numerous eye abnormalities (reviewed in Georgala et al., 2011). Similarly, defects in retinal proliferation and proper differentiation of rod photoreceptors are seen with conditional loss of $Rb$ in retinal progenitors (Donovan and Dyer, 2004).

We have evidence that $Rb$ and Pax6 interact genetically during cortical development, and that this interaction occurs, at least in part, through cell-autonomous mechanisms. We observed a strong correlation of high $Rb$ expression in Pax6 cortical progenitors. Furthermore, we observed increased Pax6 expression in Pax6+ progenitors with loss of $Rb$. An indirect mechanism by which $Rb$ family members could affect $Rb$ function is through direct regulation of Pax6 expression. We showed that there is a conserved E2F site within the Pax6 P1 promoter, and that $Rb$ family members can bind to the Pax6 promoter in E14.5 cortical extracts, using primers that flank this conserved E2F site. Pax6 levels increase with loss of $E2f1$, $E2f2$, and $E2f3a$ within the lens (Wenzel et al. 2011), indicating that E2F regulation of
Figure 5-1: Pax6 and Rb function in similar tissues in both mouse and humans

The expression of Pax6 and Rb, as determined by in situ hybridization, overlap during the development of the mouse cortex (A). The loss of function of Pax6 or Rb leads to defects in many of the same tissues, both in humans and in mouse (B), indicating that they may be functioning within the same genetic pathways. In situ hybridization images from Allen Developing Mouse Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009.

Pax6 indeed may be occurring. Then again, the presence of a Pax6 binding site within 30bp of this conserved E2F site raises the intriguing possibility that Rb family members could be directly interacting with Pax6 (see below) to regulate Pax6 expression. To determine whether Rb family members regulate Pax6 transcriptionally, analysis of Pax6 mRNA by either RT-PCR or in-situ hybridization in the Rb family deficiencies would be required.

Alternatively, the strong expression of Rb in Pax6+ progenitors may be part of a mechanism by which pRB regulates Pax6 transcriptional activity through a protein-protein interaction. Many Pax family members, including Pax6, contain a paired-like homeodomain, which can mediate an interaction with Rb family members. All three pocket proteins can bind with Pax3 and Pax8 (Wiggan et al., 1998; Miccadei et al., 2005), while pRB can bind Pax5 (Eberhand and Busslinger, 1999). Complexes of Pax6 and hypophosphorylated pRB were shown in chicken lens extracts by co-immunoprecipitation as well as in vitro pull down assays (Cvekl et al., 1999). These interactions are thought to modulate Pax transcriptional activity, so that pRB acts as a repressor of Pax3- (Wiggan et al., 1998), Pax2- (Yuan et al., 2002), and Pax6- (Cvekl et al., 2004) mediated gene activation. Interestingly, activation of the alpha-A-crystallin promoter by Pax6, which is needed for proper lens development, is repressed by the addition of pRB (Cvekl et al., 2004). If a similar interaction is seen within cortical precursors, then as the expression of Rb is turned on during corticogenesis, pRB may be able to modulate Pax6 transcriptional activity.

Regulation of Rb expression already is known to be important for the regulation of cell cycle exit and developmental genes during myogenesis. The bHLH factor, MyoD, induces Rb expression during myogenesis, and Rb promotes MyoD activation of genes necessary for late stages of muscle differentiation (Novitch et al., 1996). Additionally, high levels of hypophosphorylated Rb are postulated to bind HDACs, and therefore displace MyoD and HDAC complexes (Puri et al., 2001). This, in turn, allows MyoD to interact with histone acetyltransferases and SWI/SNF complexes to activate genes needed for differentiation. There may be a similar situation in cortical progenitors, where as Rb expression is turned on in Pax6 progenitors, interactions between Rb and Pax6 modulates Pax6 transcriptional activity. This, in turn, may promote the activation of genes needed for neurogenesis, which are known targets of Pax6 (reviewed in Osumi et al., 2008).
Similar to pRB, Pax6 is a regulator of genes necessary for development, as well as cell cycle progression. Since Pax6 controls the maintenance of the progenitor state as well as promoting neurogenesis, it has both positive and negative effects on the cell cycle. Pax6 deficiency leads to decreases in p27, p21, and p57 in the E12.5 cortex, and directly regulates p27 expression (Duparc et al., 2007), indicating that Pax6 negatively regulates the cell cycle. Alternatively, overexpression of Pax6 in the E14.5 cortex leads to increased expression of the positive cell cycle regulators Cdk4, Ccnd2, and Ccne1 (Sansom et al., 2009). Both the loss of Pax6 activity in the Pax6 Sey/Sey mouse, and the overexpression of Pax6 in the Pax77 mouse, lead to a decrease in cortical size. This is due to increased cell cycle exit of progenitor cells, although the loss of Pax6 affects early progenitors at E12.5, while overexpression of Pax6 affects late progenitors at E15.5 (Quinn et al., 2007, Manuel et al., 2007). The binary functions of Pax6 in cell cycle control and differentiation are context specific, so while loss of Pax6 reduces the neurogenic potential of radial glial cells taking from E14 cortex (Heins et al., 2002), Pax6 is required for proliferation of progenitors at E12.5 (Quinn et al. 2007). Since RbP-LacZ expression increases as corticogenesis progresses in the embryo and neurogenesis advances in primary cultures, it would be interesting to know if the timing of the Rb expression modulates how Pax6 regulates cell cycle exit and Pax6+ progenitor decisions to renew or differentiate. Since Pax6 can regulate both positive and negative upstream regulators of pRB function, regulation of Rb at the transcriptional level, as well as post-translational level, is most likely occurring in Pax6+ progenitors.

**Rb Family Members Vary in Function During Neuronal Development**

At the initiation of this work, we expected that loss of the Rb family members would lead to an increase in expression of the RbP-LacZ reporter, since Rb family members are traditionally known to function as repressors. The opposing effects of Rb loss vs. dual loss of p107 and p130 on RbP-LacZ activity underscores the fact that the functions of the pocket proteins within the developing nervous system are not completely redundant. Uncovering how pocket protein antagonism occurs could lead to a better understanding of the different functions of these pocket proteins. Interestingly, a similar result has been seen using a GFP BAC reporter of Rb expression (RbP-eGFP contains ~50kb upstream and 100kb downstream of the Rb start site, where loss of Rb or p107 led to an increase and loss of p130 led to a decrease of Rb-eGFP activity in adult myeloid cells (Burkhart et al., 2010a). Interestingly, there was no
change in other hematopoietic cell types, like B cells and T cells, similar to our study where we see changes in RbP-LacZ activity restricted to Pax6 progenitors.

There is some evidence that the pocket proteins do not share all functions. For example, while Rb loss has been found to inhibit adipogenic differentiation of 3T3 cells, loss of p107 and p130 actually increases differentiation (Classon et al., 2000a), indicating that, although structurally similar, these proteins have distinct cellular functions. We see similar differences in neuronal development, where loss of p107 or p130 leads to decreased proliferation while loss of Rb leads to increased proliferation. The decrease in proliferation in the p107- or p130-deficient cortex also contrasts with the accepted roles of these pocket proteins in cell cycle control from tissue culture experiments. A decrease in proliferation could be due to cell cycle arrest or to an inability of cells to divide. Alternatively, a decrease in proliferation could be a result of a decreased progenitor pool due to increased cell cycle exit. It has been reported that loss of p107 is important for maintenance of neuronal progenitors (Vanderluit et al., 2007), although in that study an increase in the proliferation was found. Although we did not see differences in proliferation in primary cortical culture with loss of p107, p130, or dual loss of p107 and p130, monitoring proliferation in Pax6 progenitors specifically might have revealed differences that were not seen in the culture as a whole. Furthermore, our results were obtained in the absence of FGF or serum, and with conditions optimized to avoid 3-dimensional neurosphere growth. Either FGF or 3-dimensional growth would change the proliferative index, and could change the effect of inactivation of various Rb family members on proliferation.

Loss of p107, p130, or dual loss of p107 and p130 led to decreased apoptosis in embryonic cortex, while conditional loss of Rb had little to no effect on apoptosis in the developing cortex. Whether this is due to a cell-autonomous increase in cell death or to non-cell-autonomous changes in the proper formation of the cortex is not known. Although we did not find any differences in p53 members at the transcriptional level, specific p53 family isoforms may fluctuate with the various Rb family deficiencies, resulting in decreased apoptosis. Another possibility for decreased apoptosis is that loss of p107 and/or p130 destabilizes E2F4 levels leading to abnormal E2F family regulation and developmental defects in the embryonic cortex, and indeed, we detected decreased E2f4 expression with loss of p107. In fact,
E2f4-deficient mice display defects in telencephalon development and self-renewal of neuronal progenitors (Ruzhynsky et al., 2007).

Rb family members have both redundant and specific functions during development. The distinct functions of the pocket proteins may be due to differences in structure, association with different E2F family members or other interactors, and/or differences in the timing and localization of their expression during development. Clearly, the opposing roles of p107 and p130 vs. pRB in the developing cortex deserve further study.

**Increase in Nuclear Size and Polyploidy with Rb-Deficiency**

Why RB acts as such a potent tumor suppressor while p107 and p130 do not has been a pressing question since all three pocket proteins can repress the expression of genes necessary for cell cycle progression. Interestingly, we found that the nuclei of Rb-deficient cortical neurons were consistently larger, and this was not seen with loss of p107 and/or p130. While we did not measure DNA content, this increase in nuclear area may be an outcome of increased ploidy with Rb loss.

In normal development, decreased expression of RB is an important part of the endoreduplication process that has been found in plants, Drosophila, and mammals (Sabelli et al., 2009). In the mouse placenta, Rb levels are significantly decreased as giant trophoblast cells undergo endocycles that are required for differentiation (Soloveva and Linzer, 2004). In cell culture, loss of Rb is known to lead to polyploidy in certain contexts. Induction of p21 in wild type MEFs leads to a cell cycle arrest in G1, but in Rb-deficient MEFs, p21 induction leads to a G2/M arrest with many cells containing 4n and 8n DNA content (Niculescu et al., 1998). Similarly, polyploidy is seen with Rb loss in mouse adult fibroblasts (MAFs) with time in culture (Mayhew et al., 2004), in which increased expression of pre-replication factors (e.g. Cdc6 and PCNA) and formation of pre-replication complexes (Srinivasan et al., 2007), lead to multiple rounds of DNA replication per cell cycle. Many of the components involved in formation of the pre-replication complex (Cdc6) as well as regulators of complex formation (CcnE1) are E2F targets, and thus, likely Rb regulated. We know that loss of Rb in E14.5 cortical extracts leads to an increase in E2f1 levels, which on its own has been shown to increase polyploidy in MAF cells (Srinivasan et al., 2007).

Interestingly, polyploidy which occurs with knockdown of pRB appears to be specific to cells undergoing senescence (Chicas et al., 2010) or differentiation (Novitch et al., 1996), and not in actively
dividing cells, implicating that pRB may regulate different target genes during different cellular states. The increase in ploidy during RAS-induced senescence is RB specific, and does not occur with knockdown of p107 or p130 (Chicas et al., 2010). In muscle differentiation, Rb-deficient myocytes positive for the MHC, a marker of early muscle differentiation, display an increase in DNA content at a greater frequency than non-differentiating myocytes (Novitch et al., 1996). This would be a similar situation to the primary cortical culture, where many of the cell types express markers of neuronal differentiation, but also display an increase in nuclear size. Although we did see an increase in EdU incorporation in Rb-deficient cortical cultures on Day 2, this difference is gone by Day 4 in culture. Whether this is due to cell cycle arrest, senescence, or differentiation with time in culture is not known. Morphologically, Rb-deficient neurons develop longer axons and express mature neuron markers with time in culture, indicating that Rb-deficient neurons can differentiate, even with an increase in ploidy.

Protection against polyploidy could be part of how RB protects against tumorigenesis. Retinomas, a benign retinal tumor found in retinoblastoma patients, show loss of both copies of RB as well as a low level of chromosomal instability, but still express markers of retinal differentiation and senescent markers like p21 (Dimaras et al., 2008). Even though additional mutational events are required for tumorigenesis, the loss of RB is an initiating step in the formation of retinoblastoma. The protection against polyploidy by RB could explain why loss of RB function occurs so frequently during tumorigenesis, but loss of p107 or p130 is rare.

**Future Directions**

To further understand how loss of p107 and p130 leads to a decrease in Rbp-LacZ activity within Pax6 progenitors, a more comprehensive examination of the potential changes at the Rb promoter is required. Using ChIP to look for potential silencing marks at histones, such as trimethylation of lysine 27 of histone H3, we could determine if the observed decrease in Rbp-LacZ activity we see is due to direct changes at the Rb promoter. Additionally, an investigation of promoter occupancy by the Rb family independent E2F family members (E2F6, E2F7, and E2F8), could help determine if the additional recruitment of a repressor leads to a decrease in Rb expression in the p107/-;p130/- cortex. Finally, to determine the DNA methylation status of the Rb promoter, bisulfite sequencing of DNA from wild type and
various Rb family member deficient cortices could be used to test if changes in Rb promoter methylation could be contributing to the decrease in RbP-LacZ activity with p107 and p130 loss.

Loss of p107 and/or p130 led to very different effects in the cortex than loss of Rb, suggesting that they may regulate different target genes within the cortex. We measured changes in gene expression of only a small set of potential E2F targets in the various Rb family member deficiencies. A non-biased analysis of gene expression in the various Rb family deficient cortices by microarray analysis could uncover genes that are differentially regulated by Rb, p107, and p130. This could help delineate how loss of Rb and dual loss of p107 and p130 can have opposing effects on RbP-LacZ activity as well as opposing effects on cortical development. Additionally, since Pax6 is potentially an E2F target, RT-PCR analysis to examine changes in Pax6 expression in cortex from the various Rb family deficiencies would be necessary.

Importantly, the determination of DNA content in Rb-deficient neurons would be essential to confirm that increased nuclear size is truly a phenotype of increased ploidy. Analysis by flow cytometry, by treatment with propidium iodide, would allow us to look for increases in DNA content with Rb deficiency. One potential obstacle for this approach is that dissociation of the mature neurons in primary cortical culture to create single cell suspensions may be unfeasible. A potential solution to this problem would be to perform the experiments on dissociated cortices using embryos at a later embryonic time point, after most neurogenesis has occurred. Alternatively, we could use the DAPI fluorescent signal intensity as a measure of DNA content from images of wild type and Rb-deficient neurons.

Since Rb and Pax6 mutant mice share many similar phenotypes, it is possible that they work within the same genetic pathway. To test if Rb and Pax6 interact genetically, crosses between conditional Rb mutant mice and conditional Pax6 mutant mice can be performed to test if loss of Pax6 can rescue any of the neuronal, retinal, and lens defects of the Rb-deficient mouse. Rescue of these phenotypes would suggest that Pax6 and Rb act antagonistically in the same genetic pathway within these tissues. Compound loss of Pax6 and Rb may rescue the differentiation defects in the Rb-deficient retina and lens, but it may not affect the ectopic proliferation seen within the Rb-deficient cortex since it occurs within regions where Pax6 is not expressed. However, the conditional loss of Rb in the cortex may rescue the
decreased cortical size and increased cell cycle exit of neuronal progenitors seen within the *Pax6 (Sey/Sey)* if they are acting within the same pathway.

Additionally, immunohistochemical analysis of Pax6 in parallel with Xgal analysis of adjacent sections of *T157* embryos and brains at various developmental time points could pinpoint when *Rb* expression is turned on in Pax6 progenitors, and whether it is maintained within the adult brain. Since potential pRB and Pax6 protein-protein interactions have been shown in the literature, it would be interesting to know if this relationship occurs within the developing cortex. Cortical extracts can be used to perform co-immunoprecipitation experiments to see if Pax6 and pRB interact. Additional co-immunoprecipitations with both p107 and p130 would be performed to see if it is a specific interaction with pRB.
Summary

Our lab has previously shown that loss of the E2F site within the critical cassette of the Rb promoter leads to deregulation of the Rb promoter in the CNS (Agromayor et al., 2006). Therefore, we proposed that Rb family members act as repressors of Rb expression in vivo, but what we found was more complicated. We discovered that RbP-LacZ activity increased in conditionally Rb-deficient backgrounds, but decreased in a p107;p130-deficient background, suggesting that these family members were working in opposing ways to regulate Rb expression during corticogenesis (Chapter 1).

Using ChIP, we found evidence that Rb family members (particularly pRB and p130) occupy the Rb promoter in the E14.5 cortex, and thus directly regulate Rb expression in vivo (Aim 2). We found modest shuffling of E2F and pRB family members within the various mutant cortices, although this did not adequately explain the differences in RbP-LacZ activity in the Rb family mutant deficiencies.

We also found that loss of Rb family members leads to changes in neuronal development that may indirectly affect RbP-LacZ activity (Aim 2). Analysis of conditionally Rb-deficient cortex led to many expected results based on the known role of Rb in regulating cell cycle progression. For instance, increased cortical size and ectopic proliferation could lead to the appearance of increased RbP-LacZ activity within the Rb-deficient cortex. Unexpectedly, loss of p107 or p130 alone led to decreases in proliferation within the ventricular and sub-ventricular zones of the cortex, which is counter to the known roles of p107 and p130 as repressors of cell cycle progression. Additionally, we found that loss of p107 and/or p130 leads to decreased apoptosis within the developing cortex, suggesting that these proteins are needed for proper cortical development. While we did not find major changes in the expression of classical neuronal regulators or E2F targets in the various Rb family mutant cortices, we did find modest deregulation of E2f family members, which could indirectly lead to changes in Rb expression (Aim 2).

Using primary cortical neuron cultures, we identified the subsets of cells expressing the RbP-LacZ reporter as either Pax6+ progenitors (the majority) or mature differentiated neurons (the minority, including calretinin+/reelin+ or calbindin+ or GAD67+ neurons) (Aim 3). The strong association between RbP-LacZ activity and Pax6+ progenitors is especially interesting due to the overlapping functions of Rb and Pax6 in cortical and retinal development. While we determined that neither the frequency nor the intensity of RbP-LacZ expressing cells changes within the total cell population in the various Rb family
mutant cultures, both the frequency and intensity of RbP-LacZ activity decreases within the Pax6+ population with dual loss of p107 and p130. Therefore, there is a cell-autonomous decrease in RbP-LacZ activity in p107- and p130-deficient Pax6+ progenitors, suggesting that p107 and p130 act together to activate Rb expression. Interestingly, we found that Rb loss leads to increased nuclear size in cortical cultures, which presumably indicates increased DNA content. However, the larger nuclei of Rb-deficient neurons would require higher RbP-LacZ activity to produce similar Xgal intensity, which may explain why we did not detect increased RbP-LacZ activity in Pax6+ progenitors from Rb-deficient primary cortical cultures.

Therefore, we conclude that regulation of the Rb promoter by Rb family members occurs in a cell-type specific fashion within the developing cortex, and is particularly important in Pax6+ progenitors (Figure 5-2). The surprising role of p107 and p130 as activators of Rb expression highlights the importance of understanding the tissue- or cell-type specific functions of proteins during development, which can be overlooked in tissue culture systems. The de-regulation of the Rb promoter in Pax6+ progenitors with loss of p107 and p130 indicates that there is a complex and antagonistic relationship between Rb family members. This suggests that regulation of Rb expression may be especially important in certain cell types during neuronal development, similar to what is seen within myogenesis. More careful analysis of the phenotypes within these Rb family member deficiencies may uncover how the functions of these proteins diverge in vivo and help explain how pRB, and not p107 and p130, plays such an prominent role in tumorigenesis.
Based on our results, we found that p107 and p130 are acting upstream of pRB and are necessary for the activation of *Rb* expression, by either leading to direct activation of the *Rb* promoter, or by indirectly affecting the Pax6 radial glia populations. pRB can repress its own activation through direct autoregulation of its own promoter. pRB is also required for the maintenance of Pax6 progenitors, at least in our primary cortical culture system, as *Rb*-deficient cultures contain less Pax6+ cells. Additionally, pRB acts to repress Pax6 function, by repressing the expression of *Pax6*, possibly through direct regulation of the *Pax6* promoter (our data), and potentially through a direct interaction between Pax6 and pRB (Cvekl et al., 2004).
Materials and Methods

Generation of Mice:

*T157 RbP-LacZ* mice were generated previously by Agromayor et al. (2006) and maintained on a C57BL/6 background. *p107* (M.H. Lee et al., 1996) and *p130* (Cobrinik et al., 1996) germline knockouts were maintained on a mixed 129Sv/C57BL/6 background. For *RbP-LacZ* analysis, *p107* and *p130* lines were crossed once to *T157* line to generate *T157; p107+/-, T157; p130+/-, and T157; p107+/-; p130-/-* mice respectively. *T157; p107* and *T157; p130* homozygous animals were generated by crossing *T157; p107+/-* and *T157; p130+/-* mice to *p107+/-* and *p130+/-* mice respectively. Double deficient *p107-/-; p130-/-* embryos were generated by crossing *T157; p107+/-; p130-/-* mice to *p107+/-; p130-/-* mice. All comparisons for double deficient mice crosses were done in a *p130* deficient background.

*RbLoxP/LoxP* (FVB; 129/OLA-Rb1tm2Brn) mice were generated by Marino et al. (2000). Mice were obtained on a FVB x129/OLA background. FVB/129 experiments were done by directly mating *RbLoxP/LoxP* mice to *T157 RbP-LacZ* mice to generate *T157; RbLoxP/LoxP* mice. Alternatively, *RbLoxP/LoxP* mice backcrossed to C57BL/6 background at either 6 or 10 generations and then crossed to *T157* line to generate *C57BL/6 T157; RbLoxP/LoxP* line. The *Meox2Cre* line developed by Tallquist and Soriano (1999) was obtained from Jackson laboratories (B6.129S4-Meox2CreSor) and maintained on a C57BL/6 background. We generated *Meox2Cre; RbLoxP/+* mice by crossing *Meox2Cre* heterozygotes to the *RbLoxP/LoxP* line either on the FVB/129 background or C57BL/6 background. Generation of *T157; Meox2-Cre; RbLoxP/LoxP* embryos was done by crossing the *Meox2-Cre; RbLoxP/+* line to *T157; RbLoxP/LoxP* line, either on a FVB/129 or C57BL/6 background.

*Nestin-Cre* mice (B6.Cg-Tg(Nes-cre)1Kln/J) generated by Tronche et al. (1999) were obtained from Jackson Laboratory and were maintained by crossing to C57BL/6 wild type animals. *Nestin-Cre* mice were crossed to C57BL/6 *RbLoxP/LoxP* line to generate *NestinCre; RbLoxP/+* line. Generation of neuronal specific conditional *Rb* null embryos were done by crossing *NestinCre; RbLoxP/+* to *T157; RbLoxP/LoxP* mice and testing for excision by PCR analysis. The ROSA26Cre reporter R26R developed by Soriano (1999) was obtained from Jackson Laboratories and maintained on a C57BL/6 background. R26R homozygotes were crossed to either *Meox2Cre* or *Nestin-Cre* lines to generate *Meox2Cre; R26R* and *NestinCre; R26R* heterozygous embryos.
For E12.5 analysis, the constitutive Rb model generated by Jacks et al. (1992) was used and we generated embryos by crossing Rb+/- heterozygous mice on the C57BL/6 background.

**Genotyping by PCR analysis**

Animals used for breeding and mating were genotyped at weaning using genomic tail DNA. Embryos were genotyped from DNA acquired from the inner yolk sacs for all crosses. Midbrain DNA or dissociated cortical cells were used to look for excision from the RbLoxP locus. Genotyping for Nestin-Cre and RbLoxP alleles were multiplexed with primers specific to the wildtype Dp1 locus to assess the abundance of genomic DNA (loading control). The T157 RbP-LacZ transgene PCR was multiplexed with primers specific for the wild type E2f1 locus or Dp1 locus to assess the abundance of genomic DNA. Primers and programs are listed in Table 6-1. Product sizes are as follows: T157: LacZ L410/L119 – 225bp; ROSA26R: LoxStopLox L275/L276 – 1146bp, Wildtype L275/L277 – 374bp; Meox2Cre: Wildtype L278/L280 – 411bp, Cre L279/L280 – 311bp; NestinCre: Cre L274/L335 – 300bp; RbLoxP: Rb18L/Rb19EL – Wildtype 230bp, LoxP 290bp; Rb18L/Rb212 – Wildtype 660bp, LoxP 730bp, Excised 260bp; p107: Wildtype L330/L331 – 280bp, Mutant L330/L340 – 220bp; p130: Wildtype L415/L326 – 249bp, Mutant L415/L327 – 338bp; Zfy1: Wildtype Zfy1/L355 – 140bp; Dp1: Wildtype L75/L78 – 180bp; Wildtype E2f1 – L31/L38 – 290bp.

Genotyping was performed using QIAGEN Multiplex PCR Kit (#206143). p107 and p130 PCRs were multiplexed for analysis of p107; p130 mice and embryos. Reactions consisted of common primers at 0.5pmol/µl, unique primers at 0.25 pmol/µl, loading control primers at 0.1 pmol/µl, and 1x Master Mix. 1X Q solution was added to the primer/mastermix stock solution for T157 reactions to improve amplification. Primers specific to the Zfy1 gene specific to the Y chromosome were added to PCRs for Meox2Cre, Nestin-Cre and p107; p130 multiplex PCRS at 0.1 pmol/µl for sex determination of embryos. Multiplex PCR reactions were run on high percentage 1x Tris Acetate EDTA (TAE) agarose gels, with 3%-4% agarose, for one (p107; p130, Meox2Cre, NestinCre, T157) or two (RbLoxP) hours using 1x TAE buffer. Genotypes were determined by visualization using 5 µg/ml ethidium bromide and band sizes were determined by comparison to a 100bp ladder (NEB N3231S). Examples of genotyping of the various lines are shown in Figure 6-1.
Figure 6-1: Determination of genotypes for T157, p107, and p130 embryos.

DNA from the inner yolk sacs of embryo litters was extracted and used for PCR analysis. Each gel represents one litter, with each embryo represented by a letter. The first lane of each gel contains 100bp ladder as a marker for DNA band size. DNA samples from the parents were used as controls. Crosses shown are for p130(A), p107 (B), p107;p130(C), and RbP-LacZ. p130 (A) and p107 (B) are run with primers for wild type p107 and p130 respectively to act as internal loading controls. Sex determination was performed by the addition of primers for Zfy, a Y-chromosome specific gene, into p107, p130, and p107;p130 PCR reactions. T157 status (D) was determined by the presence of RbP-LacZ using primers specific to E2f1 to act as a loading control.
<table>
<thead>
<tr>
<th>Line</th>
<th>Primer</th>
<th>Location</th>
<th>Sequence</th>
<th>Protocol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T157</td>
<td>L410</td>
<td>LacZ</td>
<td>5’-AAGGGGGATGTGCTGCAAGGCAGAT-3’</td>
<td>94°C 1 min, 54°C 90 sec 40 cycles, 72°C 1 min</td>
<td>Modified from Agromayor et al 2006</td>
</tr>
<tr>
<td></td>
<td>L119</td>
<td>Rb promoter</td>
<td>5’-TTGTAACGGAGTCGGGTGAGG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROSA26R</td>
<td>L275</td>
<td>ROSA26R for</td>
<td>5’-CGCTTAAAGGCTAAGCCTGAGTGTG-3’</td>
<td>94°C 30 sec, 62°C 1 min 36 cycles, 72°C 90 sec</td>
<td>Soriano 1999</td>
</tr>
<tr>
<td></td>
<td>L276</td>
<td>SAβgeo</td>
<td>5’-GCCAAGAAGTTTGCTCCTCAACC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L277</td>
<td>ROSA26R rev</td>
<td>5’-GGGAGCCAGGAAATGGATATG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meox2Cre</td>
<td>L278</td>
<td>MORE rev</td>
<td>5’-AAGGTGTGAGAGTGTCGGGTGATT-3’</td>
<td>94°C 1 min, 64°C 1 min 36 cycles, 72°C 1 min</td>
<td>Tallquist &amp; Soriano 1999</td>
</tr>
<tr>
<td></td>
<td>L279</td>
<td>MORE for</td>
<td>5’-GGGACCACCTCTCTGCGTTTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L280</td>
<td>MORE Cre</td>
<td>5’-CCAGATCCTCTCAGAAATCAGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NestinCre</td>
<td>L274</td>
<td>Cre forward</td>
<td>5’-GTGAAACAGCATTGCTGTCACTT</td>
<td>94°C 1 min, 60°C 1 min 30 cycles, 72°C 1 min</td>
<td>Graus-Porta et al 2001</td>
</tr>
<tr>
<td></td>
<td>L335</td>
<td>Cre reverse</td>
<td>5’-GACATGGTCCAGGGCAAGCCGGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RbloxP</td>
<td>Rb18L</td>
<td>Rb WT</td>
<td>5’-GGGCGTGTCCTCATCAATG-3’</td>
<td>94°C 1 min, 58°C 1 min 35 cycles, 72°C 1 min</td>
<td>Modified from Marino et al 2000</td>
</tr>
<tr>
<td></td>
<td>Rb19EL</td>
<td>Rb loxP</td>
<td>5’-CCCTCTCAAGGCTGACTCAGTGGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Rb212</td>
<td>Rbα19</td>
<td>5’-GAAAGGAAAGGCCTACGGATGGG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p107</td>
<td>L330</td>
<td>Common</td>
<td>5’-TCGGTGCATCTGCTGACAG-3’</td>
<td>94°C 1 min, 56°C 1 min 35 cycles, 72°C 1 min</td>
<td>Modified from Lee et al 1996</td>
</tr>
<tr>
<td></td>
<td>L331</td>
<td>WT</td>
<td>5’-GTCCTGACATGAAAACAAGAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L340</td>
<td>Neo</td>
<td>5’-GGTTGCGCTACCGGGATGGT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p130</td>
<td>L326</td>
<td>WT</td>
<td>5’-ACGGATGTCAGTGCACG-3’</td>
<td>94°C 1 min, 56°C 1 min 35 cycles, 72°C 1 min</td>
<td>Modified from Cobrinik et al 1996</td>
</tr>
<tr>
<td></td>
<td>L327</td>
<td>Neo</td>
<td>5’-TACATGGTTCCTCTTCCAGG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>L415</td>
<td>Common</td>
<td>5’-GACACAGTCCATGCAGAGAC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zfy1</td>
<td>Zfy1</td>
<td>Forward</td>
<td>5’GAGTAGACATTGCACTTTGCACG-3’</td>
<td>Varied</td>
<td>S. Leung and M. Kohn</td>
</tr>
<tr>
<td></td>
<td>L355</td>
<td>Reverse</td>
<td>5’-GGAGGTATGGAGTACATGAGAGT-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dp1</td>
<td>L75</td>
<td>Intron3</td>
<td>5’-GACACGTCTGATTGTTGAATT-3’</td>
<td>Control for NestinCre and RbloxP</td>
<td>Kohn et al, 2003</td>
</tr>
<tr>
<td></td>
<td>L78</td>
<td>Exon4</td>
<td>5’-ACTGGGTTGGGCTGTCACCC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2f1</td>
<td>L31</td>
<td>Intron 3</td>
<td>5’-GCTGGATTGGTGTCAGACAC-3’</td>
<td>Control for T157 Rbp-LacZ</td>
<td>Yamasaki et al 1998</td>
</tr>
<tr>
<td></td>
<td>L38</td>
<td>Exon2</td>
<td>5’-CCCCACAGCTGGGAAACCAAGGT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Embryo Collection**

Stud males were mated with mature females overnight and the presence of a vaginal plug was determined as Day 0.5. Pregnant females were sacrificed on embryonic days E14.5, and E16.5. Euthanasia was performed by carbon dioxide exposure for two minutes followed by cervical dislocation. Embryos were removed from the uterine horn and inner yolk sacs were taken for genotyping. Embryos were dissected into PBS pH 7.4 (phosphate buffered saline, Invitrogen# 21600010) and directly placed in 3.7% formaldehyde in PBS pH 7.4 for one hour prior to Xgal processing.

**Tissue Collection**

Adult brains were dissected out at 8 and 12 weeks from T157 wild type mice. Stud males were mated either overnight (to generate E12.5 and E14.5 embryos) or for 4-6 hours (to generate E15 embryos) to mature females. The presence of vaginal plug was assumed to be either 0.5 days (overnight mating) or Day 0 (short mating). Embryos were removed of their extra embryonic membranes and either processed for whole mount analysis or brains were micro-dissected prior to fixation. For E12.5 analysis, both the telencephalic and diencephalic regions were collected. For E14.5 analysis, the two lobes of the cortex were removed from the remaining developing brain.

**Xgal Staining of Whole Mount Embryos**

E14.5 embryos were fixed in 3.7% formaldehyde in 1x PBS pH 7.4 for 1 hour at 4°C. Embryos were washed three times in PBS pH7.4 and then stained in Xgal solution at 30°C overnight for 14-18 hours. Xgal solution consisted of 1mg/ml of Xgal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside, Roche 10745740001) dissolved in DMSO, 2 mM MgCl₂, 0.02% NP-40, 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆ dissolved in PBS pH7.4. Embryos were washed three times in PBS after staining and post-fixed in 3.7% formaldehyde in 1x PBS for > 24hours. Embryos were transferred to PBS and photographed for analysis. To test for excision from the RbLoxP locus, brains were removed after photography and genomic DNA was obtained for PCR analysis.

**Xgal Staining of Whole Mount Brains**

Adult brains were dissected out and sliced into 3mm coronal slices using a 1mm brain matrix. Slices were fixed in in 3.7% formaldehyde in 1x PBS at 4°C for 30 minutes, washed with PBS, and stained in Xgal solution at 37°C for 3 hours. Photos were taken after washing and post fixation in 3.7%
formaldehyde in 1x PBS for 72 hours. Embryonic brains were micro-dissected out and fixed on ice in 3.7% formaldehyde in 1x PBS for 20 minutes. Brains were washed with PBS and stained for 4 hours (E15.5 and E16.5) or 6 hours (E13.5 and E14.5) in Xgal solution at 30°C. Brains were washed with PBS, post fixed in 3.7% formaldehyde in 1x PBS, and cortices were removed from the midbrain for photographic analysis. Xgal solution consisted of 1 mg/ml of Xgal (dissolved in either DMSO or DMF), 0.02% NP40, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, and 2 mM MgCl₂ in PBS pH 7.4.

BrdU Incorporation

Pregnant females were intraperitoneally injected with 10 mg/ml of 5-bromo-2'-deoxyuridine (BrdU Sigma B9285) and 0.6 mg/ml 5-Fluoro-2'-deoxyuridine (Sigma F-0503) in PBS. Females were sacrificed 1 hour after injection by carbon dioxide exposure for 2 minutes followed by cervical dislocation, and embryos were removed from extraembryonic tissues and fixed in 3.7% formaldehyde in PBS for four hours. Embryos were processed and embedded in paraffin blocks. Fifty 5-micron double sagittal sections were taken of each embryo with analysis done on at least every 5th section using a BrdU staining kit (Invitrogen 93-3943). Sections were de-paraffinized in xylene, and endogenous peroxidase activity was quenched by a 10 minute treatment in 3% H₂O₂ in methanol. Embryonic sections from the p130 and p107;p130 lines were brought to a boil three times in 10 mM citrate buffer pH 6.0 to improve antigen retrieval. Tissues were then treated as per kit’s instructions. Tissues were trypsinized for 10 minutes and denatured for 30 minutes. Tissues were blocked and the biotinylated anti-BrdU antibody was applied for 1 hour. Antibody was omitted on adjacent sections to act as a negative control. Streptavidin-peroxidase was applied for 10 minutes and chromagen detection was performed using 3,3'-Diaminobenzidine (DAB) for 10 minutes and hematoxylin counterstain for 30 seconds. Sections were dehydrated through ethanol and mounted in Permount (Fisher Scientific SP15-100) for analysis by light microscopy. All washes were done using PBS.

TUNEL Analysis

The TUNEL assay for apoptosis was performed on every 4th section of 50 5-micron double sagittal sections using the Roche In Situ Cell Death Detection Kit (Cat. No. 11684817910). Sections were deparaffinized in xylene and proteinase K digestion at 5mg/ml in PBS was performed for 15 minutes. Endogenous peroxidase activity was quenched by 15 minute treatment in 3% H₂O₂ in methanol.
Embryonic sections from the \textit{p130} and \textit{p107}; \textit{p130} lines were brought to a boil three times in 10mM citrate buffer pH 6.0 to improve signal. Tissues were blocked using 3% bovine serum albumin (BSA) (Sigma A9418) in PBS for 20 minutes. Sections were incubated in TdT enzyme solution for 1 hour at 30°C, with enzyme omitted from the adjacent section to use as a negative control. Tissues were then incubated with the Converter-POD peroxidase for 30 minutes at 30°C, and chromagen detection was performed using DAB (10min) and hematoxylin counterstain (30 seconds). Sections were dehydrated through ethanol and mounted in Permount for analysis by light microscopy.

**Quantitative RT-PCR Analysis**

Total RNA was isolated from E12.5 brains and E14.5 neocortex using Trizol reagent (Invitrogen 15596-026). cDNA was reverse transcribed from 5 µg of total RNA using Moloney murine leukemia virus reverse transcriptase at 200 units per reaction (Promega M170A) for one hour. Real-time reverse transcription-PCR (RT-PCR) was performed using specific RT-PCR primer pairs that are commercially available from Qiagen (actin, \textit{Rb}, \textit{p107}, \textit{p130}, \textit{E2f1}, \textit{E2f2}, \textit{E2f3}, \textit{E2f4}, \textit{E2f5}, \textit{E2f6}, \textit{E2f7}, \textit{E2f8,Fgf1}, \textit{Fgf2}, \textit{Fgfr1}, \textit{Fgfr2}, \textit{Fgfr3}, \textit{Fgfr4}, \textit{Hes5}, \textit{Hes6}, \textit{p63}, \textit{p73}, \textit{Bbc3(PUMA)}, \textit{Mcl1}) or from Superarray (\textit{Dp1}, \textit{Dp2}, \textit{E2f1}, \textit{Hes1}, \textit{p53}) (Table 6-2). Reactions were run using QuantiTect SYBR Green PCR Kit (Qiagen) on the Chromo4 Real-Time PCR System. The efficiency of amplification was established for each primer set using 10-fold serial dilutions of cDNA, and each sample was run in triplicate at a 1:10 cDNA dilution. Amplification was performed using the following program: 95°C 15 minutes, Denaturation: 94°C 15 seconds, Annealing: 55°C 30 seconds, Extension: 72°C 30 seconds, 40 cycles. Analysis was done by comparison to a standard curve established for each primer set. Values were calculated by normalizing copy number to an endogenous actin control. To compare genotypes, data was expressed as a ratio of \textit{Rb} family member(s) deficient/wild type. Significance testing was performed using the student’s t-test.

**Chromatin Preparation from Embryonic Cortex**

Pregnant females were sacrificed on E14.5 by carbon dioxide exposure for two minutes, followed by cervical dislocation. Embryos were removed from the uterine horn and dissected into PBS pH7.4. Brains were removed from the embryos, and the cortical lobes were separated from the rest of the midbrain structure. Cortices were dissociated in 500 µl dissociation medium with papain as will be described for neuronal culture. Papain digestion was inhibited by addition of 500 µl of DMEM
### Table 6-2 Primers for quantitative RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Amplified Region</th>
<th>Company</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Exons 1/2</td>
<td>Qiagen</td>
<td>QT01136772</td>
</tr>
<tr>
<td>Rb1</td>
<td>Exons 18/19</td>
<td>Qiagen</td>
<td>QT00164255</td>
</tr>
<tr>
<td>Rbl1 (p107)</td>
<td>Exons 19/20</td>
<td>Qiagen</td>
<td>QT00153258</td>
</tr>
<tr>
<td>Rbl2 (p130)</td>
<td>Exons 8/9</td>
<td>Qiagen</td>
<td>QT00143780</td>
</tr>
<tr>
<td>E2f1</td>
<td>Exon 4</td>
<td>Qiagen</td>
<td>QT01079106</td>
</tr>
<tr>
<td>E2f1</td>
<td>Exon 7 (Ref. Position 2362)</td>
<td>SuperArray</td>
<td>PPM02892A</td>
</tr>
<tr>
<td>E2f2</td>
<td>Exons 5/6</td>
<td>Qiagen</td>
<td>QT00163457</td>
</tr>
<tr>
<td>E2f3</td>
<td>Exons 2/3</td>
<td>Qiagen</td>
<td>QT01566054</td>
</tr>
<tr>
<td>E2f4</td>
<td>Exons 7/8/9</td>
<td>Qiagen</td>
<td>QT00118545</td>
</tr>
<tr>
<td>E2f5</td>
<td>Exons 4/5/6</td>
<td>Qiagen</td>
<td>QT00136829</td>
</tr>
<tr>
<td>E2f6</td>
<td>Exons 5/6</td>
<td>Qiagen</td>
<td>QT00106974</td>
</tr>
<tr>
<td>E2f7</td>
<td>Exons 5/6</td>
<td>Qiagen</td>
<td>QT00103446</td>
</tr>
<tr>
<td>E2f8</td>
<td>Exons 2/3</td>
<td>Qiagen</td>
<td>QT01063538</td>
</tr>
<tr>
<td>Dp1</td>
<td>Exon 11 (Ref. Position 1626)</td>
<td>SuperArray</td>
<td>PPM03468A</td>
</tr>
<tr>
<td>Dp2</td>
<td>Exon 8 (Ref. Position 857)</td>
<td>SuperArray</td>
<td>PPM03462A</td>
</tr>
<tr>
<td>Fgf1</td>
<td>Exons 3/4</td>
<td>Qiagen</td>
<td>QT00149296</td>
</tr>
<tr>
<td>Fgf2</td>
<td>Exons 2/3</td>
<td>Qiagen</td>
<td>QT00128135</td>
</tr>
<tr>
<td>Fgfr1</td>
<td>Exons 8/9</td>
<td>Qiagen</td>
<td>QT00198548</td>
</tr>
<tr>
<td>Fgfr2</td>
<td>Exons 2/3</td>
<td>Qiagen</td>
<td>QT00109172</td>
</tr>
<tr>
<td>Fgfr3</td>
<td>Exons 5/6</td>
<td>Qiagen</td>
<td>QT00107828</td>
</tr>
<tr>
<td>Fgfr4</td>
<td>Exons 11/12</td>
<td>Qiagen</td>
<td>QT00164346</td>
</tr>
<tr>
<td>Hes1</td>
<td>Exon 4 (Ref. Position 986)</td>
<td>SuperArray</td>
<td>PPM05647A</td>
</tr>
<tr>
<td>Hes5</td>
<td>Exon 3</td>
<td>Qiagen</td>
<td>QT00268044</td>
</tr>
<tr>
<td>Hes6</td>
<td>Exon 4</td>
<td>Qiagen</td>
<td>QT00295960</td>
</tr>
<tr>
<td>p53</td>
<td>Exon 11 (Ref. Position 1522)</td>
<td>SuperArray</td>
<td>PPM02931A</td>
</tr>
<tr>
<td>p63</td>
<td>Exons 11/12</td>
<td>Qiagen</td>
<td>QT00197904</td>
</tr>
<tr>
<td>p73</td>
<td>Exons 3/4</td>
<td>Qiagen</td>
<td>QT00123487</td>
</tr>
<tr>
<td>Bbc3 (PUMA)</td>
<td>Exons 2/3</td>
<td>Qiagen</td>
<td>QT00106638</td>
</tr>
<tr>
<td>Mcl1</td>
<td>Exons 3/4</td>
<td>Qiagen</td>
<td>QT00107436</td>
</tr>
</tbody>
</table>

Ref. Position refers to a position contained within the sequence of the amplicon based on the transcript sequence.
(Invitrogen 11960-044) with 10% fetal bovine serum. Cells were filtered through 35 µm filter cap tube (BD 352235). Cells were put on ice and cell counts using trypan blue exclusion were performed. Approximately, 7x10^6 – 9x10^6 cells were obtained from each E14.5 cortex. Cells were transferred to a 1.5ml tube and spun at 1000rpm for 5 minutes to remove media. Cells were suspended in cold DMEM/10% fetal bovine serum at 1.64x10^4 cells/µl and left on ice for 10 minutes. Volumes for each cortex were maintained between 500 and 1000 µl. The crosslinking protocol was modified from Ren and Dynlacht (2003). Crosslinking solution (11% formaldehyde, 0.1 M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, 50 mM HEPES, pH 8.0) was added to each dissociated cortex at one-tenth volume and cells were lightly shaken for 10 minutes at 4°C. The reaction was stopped by addition of one-twentieth volume of 2.5 M glycine. Cells were washed twice in 1 ml of cold phosphate buffered saline with protease inhibitor cocktail (Sigma P8340). Dry pellets were kept at -80°C until immunoprecipitation was performed.

Thawed cell pellets were suspended in SDS lysis buffer (Millipore 20-163) at 1.3x10^4 cells/µl (approximately 700-900µl per cortex) and incubated on ice for 10 minutes. Cells were sonicated at 65% output for 2 minutes in 20-second pulses using a Branson Sonifier 450 with cup horn attachment. Cells were pulsed for 20 seconds followed by 30 seconds on ice. The chromatin concentration was adjusted with SDS lysis buffer to 1.0x10^6 cells/µl. Chromatin was cleared by centrifugation and 1.0x10^6 cells/ml were used per each immunoprecipitation, which was approximately 1ml per immunoprecipitation.

Chromatin size post-sonication was determined by removing 50 µl of the sheared chromatin from the total cortex sample and reversing crosslinks by addition of 0.2 M NaCl and heating at 65°C for at least 4 hours. Chromatin was precipitated using isopropanol and resuspended in 50 µl 1x Tris-EDTA. Dilutions of chromatin were run on a 1% TAE agarose gel for 60 minutes and gels were visualized by ethidium bromide as previously described. The range of chromatin size was determined by use of both a 100bp marker and µl DNA-BstEII Digest marker (NEB N3014S), and optimization of sonication was performed so that the chromatin size was between 200bp - 1000bp.

Chromatin Preparation from Adult Cortex

Animals were sacrificed by carbon dioxide exposure for five minutes followed by thoracotomy. Each four to six week cortex was removed from the rest of the brain tissues and dissected into PBS. Cortical lobes were minced with a razor blade for 90 seconds and transferred into a 5 ml tube with 4ml of
DMEM with 10% fetal bovine serum. Tissue was further dissociated by trituration through progressively smaller bore 1ml plastic pipette tips. Crosslinking solution was added at one-tenth volume and cells were lightly shaken for 10 minutes at 4°C. The reaction was stopped by addition of one-twentieth volume of 2.5 M glycine. Cells were washed twice in 1 ml of cold phosphate buffered saline with protease inhibitor cocktail (Sigma P8340). Cells were suspended in 500 µl of SDS lysis buffer and incubated on ice for 10 minutes. Cells were sonicated at 80% output for 4 minutes in 20 second pulses using a Branson Sonifier 450 with cup horn attachment. Cells were kept on ice for 30 seconds in between pulses. Chromatin was cleared by centrifugation and one-fifth of a cortex was used per each immunoprecipitation. Shearing of chromatin was analyzed as described for embryonic cortex.

**Chromatin Immunoprecipitation**

Chromatin immunoprecipitation was performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit (Millipore 17-295) with modifications from the given protocol. Chromatin was diluted ten fold in ChIP dilution buffer with protease inhibitor cocktail (1/100) and one-hundredth volume was removed for input samples (50-70 µl). Chromatin was precleared using Protein A Agarose/Salmon Sperm DNA beads for one hour at 4°C. Supernatant was removed and chromatin was divided for individual immunoprecipitations. Antibody was added at 2 µg and immunoprecipitation was performed overnight at 4°C for 14-17 hours. Antibodies are provided in Table 6-3. Antibody/chromatin complexes were collected by addition of 20 µL of Protein A Agarose/Salmon Sperm DNA (50% Slurry) for one hour. Agarose pellets were washed with Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer, LiCl Immune Complex Wash Buffer, and two TE buffer washes for 5 minutes at 4°C. Immunoprecipitated chromatin complexes were eluted by two successive additions of fresh 1% SDS/ 0.1M NaHCO₃ and shaking for 15 minutes at room temperature. Crosslinks were reversed by addition of NaCl to a final concentration of 0.2 M and incubation at 65°C for four hours. Protein was removed by addition of 5 mg/ml proteinase K in TE (pH 6.0) to a final concentration of 0.3 mg/ml and incubation at 45°C for one hour. DNA was isolated by phenol chloroform extraction followed by ethanol precipitation with addition of 0.5 µl glycogen (Sigma G8751). DNA was suspended in 20 µl of TE and left overnight at 4°C.
### Table 6-3: Antibodies used for ChIP

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Company</th>
<th>Catalog #</th>
</tr>
</thead>
<tbody>
<tr>
<td>rlgG</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-2027</td>
</tr>
<tr>
<td>Rb-1 (C-15)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-50x</td>
</tr>
<tr>
<td>Rb-2 (M-153)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-7905</td>
</tr>
<tr>
<td>p107 (C-18)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-318x</td>
</tr>
<tr>
<td>p130 (C-20)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-317x</td>
</tr>
<tr>
<td>E2F1(C-20)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-193x</td>
</tr>
<tr>
<td>E2F2(C-20)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-633x</td>
</tr>
<tr>
<td>E2F4(C-21)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-866x</td>
</tr>
<tr>
<td>E2F6(M-40)</td>
<td>Rabbit</td>
<td>Santa Cruz</td>
<td>sc-22824</td>
</tr>
<tr>
<td>Acetyl Histone H3</td>
<td>Rabbit</td>
<td>Millipore</td>
<td>06-699</td>
</tr>
<tr>
<td>Histone H3 (trimethyl K9)</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab8898</td>
</tr>
<tr>
<td>Histone H3 (trimethyl K4)</td>
<td>Rabbit</td>
<td>Abcam</td>
<td>ab8580</td>
</tr>
</tbody>
</table>

### Table 6-4: Primers and protocols used for ChIP analysis

<table>
<thead>
<tr>
<th>Promoter</th>
<th>Primers</th>
<th>Sequence(5'-3')</th>
<th>Protocol</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb</td>
<td>RbChIP 5</td>
<td>GAGAAACCATTATGAAAGGAGGC</td>
<td>95°C 1min, 57°C 1min 36-42 cycles, 72°C 1min</td>
<td>Yamasaki Lab</td>
</tr>
<tr>
<td></td>
<td>RbChIP 6</td>
<td>GCCATTGTTGGGCTTCTGGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RbChIP 8</td>
<td>AGCCCGGCTCTGGAGAAACG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RbChIP10</td>
<td>TCTCCAGAAGGCACCAATGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intron 3</td>
<td>L356</td>
<td>CTCTGCTGGGATCAGTTAATGTT</td>
<td>95°C 1min, 60°C 1min 36 cycles, 72°C 1min</td>
<td>Developed by S.Leung</td>
</tr>
<tr>
<td></td>
<td>L360</td>
<td>GTCCACGGGTCAAGCCAGGGATC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cdc2</td>
<td>Cdc2ChIP1</td>
<td>ACAGAGCTCAGAGTCAGTTGGC</td>
<td>95°C 1min, 57°C 1min 36 cycles, 72°C 1min</td>
<td>Rayman et al. 2002</td>
</tr>
<tr>
<td></td>
<td>Cdc2ChIP2</td>
<td>CGCCAATCCAGTGCACTGAGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dhfr</td>
<td>dhfrChIP1</td>
<td>GCCTAAGCTGCGCAAGTGTAT</td>
<td>95°C 1min, 57°C 1min 36 cycles, 72°C 1min</td>
<td>Daury et al. 2006</td>
</tr>
<tr>
<td></td>
<td>dhfrChIP2</td>
<td>GTCTCCGTTCTGGCAATTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p107</td>
<td>p107ChIP1</td>
<td>GGTCCTACCTTCTATCCCTCCG</td>
<td>95°C 1min, 60°C 1min 36-42 cycles, 72°C 1min</td>
<td>Burkhart et al. 2010</td>
</tr>
<tr>
<td></td>
<td>p107ChIP3</td>
<td>GGCTTCCGTTTTCTTTTCCCTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pax6</td>
<td>Pax6ChIP3</td>
<td>CCCCAGGCGTCTTGCATTGATAGCA</td>
<td>95°C 1min, 57°C 1min 36 cycles, 72°C 1min</td>
<td>Yamasaki Lab</td>
</tr>
<tr>
<td></td>
<td>Pax6ChIP4</td>
<td>CCTCGCCTCCACCGCTTCTCAGT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Amplification of ChIP DNA**

Conventional PCR analysis was performed on ChIP samples as follows. Precipitated chromatin recovered from each input sample was diluted ten fold. For Rb promoter and p107 promoter, one tenth of precipitated chromatin from each IP was added per each reaction (2 µl). For Intron3, Cdc2, Dhfr, and Pax6 promoters, 1/20 of precipitated chromatin was added to each reaction (1 µl). PCR reactions were run using QIAGEN Multiplex PCR Kit (#206143) and 0.5 pmol/µl of each primer. Q solution (Qiagen) was added at 1x to Rb and p107 reactions because of the high GC content of the amplified regions. PCR primers and protocols are provided in Table 6-4. PCR products were run on a 1.6% TAE agarose gel for 40 minutes at 110 volts and bands were visualized by ethidium bromide as described. Band sizes for the various reactions are as follows: Rb promoter: RbChIP5/RbChIP6 – 250bp, RbChIP8/RbChIP10 - 145bp; Rb Intron 3: L356/L360 – 208bp; Cdc2 promoter: Cdc2ChIP1/Cdc2ChIP2 – 150bp; Dhfr promoter: dhfrChIP1/dhfrChIP2 – 170bp; p107 promoter: p107ChIP1/p107ChIP3 – 189bp; Pax6 promoter: Pax6ChIP3/Pax6ChIP4 – 208bp.

**Primary Cortical Neuron Culture**

Pregnant females were sacrificed on E14.5 as previously described. Embryos were removed from the uterine horn and washed in PBS pH7.4. Inner yolk sacs were removed for genotyping as previously described. To determine gender of embryos, testes or ovaries were micro-dissected at E14.5. Brains were micro-dissected and the cortical lobes were removed from the rest of the midbrain. To confirm RbP-LacZ status, midbrains were placed in Xgal solution and stained at 37°C for > 1 hour. Cortical lobes were rinsed in sterile PBS pH7.2 and placed in 250 µl of Dissociation medium prior to separation. Dissociation medium (Hutton and Pevny, 2008) consisted of: 98 mM Na₂SO₄, 30 mM K₂SO₄, 5.8 mM MgCl₂, 0.25 mM CaCl₂, 1 mM HEPES(pH 7.4), 20 mM glucose, and 0.001% Phenol Red. Media was adjusted to pH ~7.4 with NaOH and 0.2 µm sterile filtered before use.

Papain solution was made fresh for each dissection and consisted of 0.32 mg/ml of L-cysteine (SIGMA C7352) dissolved in dissociation media (pH readjusted to pH 7.4), 0.001 mg/ml (0.03 Units) papain (Roche #10108014001), and 0.33 µg/ml of DNAse (Sigma D4513 3198 kunitz/mg). Two hundred and fifty microliters of papain solution was added to the cortices and they were incubated at 37°C for 5 minutes. Cortices were triturated through a cut P200 micropipette tip 15 times and incubated for 5
minutes at 37°C. Trituration was repeated two more times using an uncut P200 micropipette tip followed by 37°C incubation for 5 minutes, for a total incubation time of 20 minutes. After a final trituration, 50 µl of trypsin inhibitor (10 mg/ml BSA (Sigma A9418) and 1 mg/ml Trypsin inhibitor (Sigma T6522), was added to inhibit digestion. Dissociated cortical cells were filtered using a 35 µm filter cap tube (BD Bioscience 352235) and 500µl of Neurobasal complete media was added to wash through remaining cells. Neurobasal complete media consists of Neurobasal Media (Invitrogen 21103-049), 1x B27 supplement (Invitrogen 17504-044), 200 mM L-glutamine, and penicillin/streptomycin at 1x (Invitrogen 15140-163). Live cells were counted using trypan blue (Invitrogen 15250-061) and cells were diluted in Neurobasal Complete media to 80,000 cells/150 µl. Cells were then plated on top of Poly-D-Lysine/Mouse Laminin 12mm coverslips (BD Bioscience 354087) by pipetting 150µl of cells onto each coverslip and incubating at 37°C for 4 hours to allow attachment. Three coverslips per cortex were then inverted in sterile 35 mm poly-styrene dishes (BD Bioscience 351008) and 2.25 ml of Neurobasal complete media was added to each dish. Cells were maintained at 37°C and 5% CO2 until day of analysis. Only cells that attached to the inverted coverslip were used for analysis. Cells were monitored for differentiation on Days 2, 4, and 6 and images were taken using a digital camera on an inverted microscope.

**Xgal Staining of Cortical Cultures**

To harvest the primary cultures, coverslips were inverted into 24 well culture plates with the addition of 500 µl of cultured media from the culture dish, so that the cells faced away from the dish. Five hundred microliters of 4% paraformaldehyde in PBS was added to each coverslip and cells were fixed for 10 minutes. Cells were then washed one time with PBS. A solution of 0.02% NP-40 in PBS was added for 2 minutes and then washed 2 times with PBS. Coverslips were again inverted and Xgal solution was added. Xgal solution was modified to contain no NP-40, 1 mg/ml Xgal dissolved in Dimethylformamide (DMF), 1 mM K₃Fe(CN)₆, 1 mM K₄Fe(CN)₆, and 2 µM MgCl₂ in PBS pH 7.4. Cells were incubated at 30°C for 15-18 hours. Cells were washed with PBS and photographed at 20x for analysis. Cell counts were done using ImageJ 1.45 (NIH). Saturation on all images was increased 50% to improve visualization. All cells that showed saturation of Xgal stain, defined as overexposure of Xgal stain as determined by visual inspection, were designated strong or high intensity. All other blue cells were designated as Xgal positive. The percentage of positive cells included all positive cells divided by the total amount of cells in a 20x
field multiplied by 100. The percentage of intense Xgal+ cells included all intense cells divided by the total number of positive cells multiplied by 100 in a 20x field. Counts were done from at least three different 20x fields. T157 RbP-LacZ negative cultures from the same dissection were stained concurrently as negative controls for staining.

**EdU Proliferation Assay**

To quantify proliferation in primary cultures, EdU uptake was monitored using the Click-it® EdU Alexa Fluor® 488 Imaging Kit (Invitrogen C10337). Coverslips were inverted into 24 well plates with 700 µl of the media from the cortical culture dish. EdU was added to a final concentration of 10 µM. A coverslip of each embryo with no EdU added was also included as a negative control. Cells were pulsed for 2 hours at 37°C. Media was then removed, and cells were fixed for 15 minutes with 4% paraformaldehyde in PBS. Cells were washed with 3%BSA diluted in PBS and permeabilized for 2 minutes with 0.02% NP-40 in PBS. Click-it® reaction buffer was added to all coverslips following product instructions. Cells were washed with PBS 3 times. Coverslips were either taken for co-staining immunofluorescent experiments as will be described or stained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen D1306 5µg/ml) diluted in PBS for 10 minutes. Coverslips were washed and mounted using ProLong® Gold (Invitrogen P36930). Analysis was done using fluorescent microscopy. Three separate 20x fields were counted using ImageJ (NIH) and all experiments were done in duplicate and averaged.

**Immunofluorescence**

To examine the different neuronal subtypes present in primary cortical culture, coverslips were inverted into 24 well plates with 700 µl of the media from the cortical culture dish. Coverslips were washed 1x with PBS, fixed for 15 minutes in 4% paraformaldehyde in PBS, and then washed and permeabilized with 0.1% NP-40 in PBS for 2 minutes. Primary and secondary antibodies used are listed in Table 6-5. For PC1/3, Pax6 (rabbit), and Sox2, cells were treated with 3.3% DMF diluted in PBS for 15 minutes to increase antigen exposure. Cells were blocked for 20 minutes. For rabbit polyclonal antibodies, cells were blocked using CAS block (Invitrogen 00-8020) and for mouse monoclonal antibodies cells were blocked in 3% BSA diluted in PBS. Primary antibodies were incubated for 1 hour at room temperature. Cells were washed and secondary antibodies were added for 20 minutes followed by DAPI nuclear stain for 10 minutes at 5 µg/ml diluted in PBS. Coverslips were washed and mounted using...
ProLong® Gold (Invitrogen P36930). Analysis was performed using digital photography and fluorescent microscopy. Counts were done using ImageJ (NIH) and percentage of positive cells for each marker was calculated using the formula: Marker+/DAPI+ multiplied by 100. For combination with EdU staining, immunofluorescent staining began with the block step after the EdU reaction and washes.

Double immunostaining of mouse and rabbit Pax6 antibodies was performed to ensure specificity of the Pax6 antibodies. Double Pax6 immunostaining was performed using the protocol for rabbit antibodies with the following changes. Both rabbit and mouse primary antibodies were incubated simultaneously as well as both secondary antibodies.

**Co-Xgal/Immunofluorescence Staining**

Day 6 cultures were treated for β-galactosidase activity as previously described for Xgal staining of primary cortical cultures except that the staining time was reduced to 4 - 4.5 hours. Immunofluorescence was performed as described with the following small changes. Coverslips were washed 1x with PBS, again inverted so that cells faced away from the culture plate, followed by two additional washes in PBS. Cells were fixed for 15 minutes in 4% paraformaldehyde in PBS, and then washed and permeabilized with 0.1% NP-40 in PBS for 2 minutes. No DMF treatment was added for PC1/3, Pax6 (rabbit), and Sox2 antibodies. Cells were blocked for 20 minutes and primary antibodies were incubated for 1 hour at room temperature. Cells were washed three times in PBS and secondary antibodies were added for 20 minutes followed by DAPI nuclear stain for 10 minutes at 5 µg/ml in PBS. Secondary antibodies for mouse monoclonal antibodies were changed to Alexa Fluor 555 rabbit anti-mouse IgG from AlexaFluor 488 goat anti-mouse IgG, due to background fluorescence created by Xgal staining. Cell numbers were counted using ImageJ (NIH) where the total number of cells was calculated by counting the number of DAPI positive cells. Double positive cells for both Xgal and marker staining was determined by overlaying photos from AlexaFluor 555, DAPI, and light images using GIMP Image Manipulation Program. The percentage of double positive cells was calculated as follows: Percent of double positive cells within the Xgal+ population = ((Marker+Xgal+) / Xgal+) multiplied by 100; Percent of double positive cells within the marker population = ((Marker+Xgal+) / Marker+) multiplied by 100.
Table 6-5: Primary and secondary antibodies used for immunofluorescence

<table>
<thead>
<tr>
<th>Primary Antibody</th>
<th>Antigen</th>
<th>Host</th>
<th>Company</th>
<th>Catalog #</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>mlIgG</td>
<td>IgG</td>
<td>mouse</td>
<td>Zymed</td>
<td>86599</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Nestin(Rat-401)</td>
<td>Nestin</td>
<td>mouse</td>
<td>Millipore</td>
<td>MAB353</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Pax6</td>
<td>Pax6</td>
<td>mouse</td>
<td>DSHB</td>
<td>Pax6(aachick1-223)</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>TuJ1</td>
<td>β-tubulin III</td>
<td>mouse</td>
<td>Covance</td>
<td>MMS-435P</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Map2a,b(AP-20)</td>
<td>Map2a / Map2b</td>
<td>mouse</td>
<td>Neomarkers</td>
<td>MS-249</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>GFAP (GA-5)</td>
<td>GFAP</td>
<td>mouse</td>
<td>Abcam</td>
<td>AB11267</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Gad67 (1G10.2)</td>
<td>Gad67</td>
<td>mouse</td>
<td>Millipore</td>
<td>MAB5406</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Reelin(G10)</td>
<td>Reelin</td>
<td>mouse</td>
<td>Millipore</td>
<td>MAB5364</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>NeuN (A60)</td>
<td>Fox-3</td>
<td>mouse</td>
<td>Millipore</td>
<td>MAB377</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>IgM</td>
<td>IgM</td>
<td>mouse</td>
<td>Sigma</td>
<td>M5909</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>O4 (81O4)</td>
<td>O4</td>
<td>mouse</td>
<td>Millipore</td>
<td>MAB345</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>PC1/3</td>
<td>PC1 / PC3</td>
<td>rabbit</td>
<td>Millipore</td>
<td>AB10553</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>Sox2</td>
<td>Sox2</td>
<td>rabbit</td>
<td>Millipore</td>
<td>AB5603</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>Pax6</td>
<td>Pax6</td>
<td>rabbit</td>
<td>Covance</td>
<td>PRB-278P</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>Calretinin</td>
<td>Calretinin</td>
<td>rabbit</td>
<td>Chemicon</td>
<td>AB5054</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>Calbindin D-28K</td>
<td>Calbindin</td>
<td>rabbit</td>
<td>Chemicon</td>
<td>AB1778</td>
<td>2µg/ml</td>
</tr>
<tr>
<td>rIgG</td>
<td>IgG</td>
<td>rabbit</td>
<td>Santa Cruz</td>
<td>sc-2027</td>
<td>1µg/ml</td>
</tr>
<tr>
<td>Tbr2</td>
<td>Tbr2</td>
<td>rabbit</td>
<td>Millipore</td>
<td>AB2283</td>
<td>1µg/ml</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary Antibodies (Invitrogen)</th>
<th>Catalog #</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexa Fluor® 555 goat anti-rabbit IgG (H+L)</td>
<td>A-21429</td>
<td>1/1500 or 1/3000</td>
</tr>
<tr>
<td>Alexa Fluor® 555 rabbit anti-mouse IgG (H+L)</td>
<td>A-21427</td>
<td>1/1000</td>
</tr>
<tr>
<td>Alexa Fluor® 488 goat anti-mouse IgG (H+L)</td>
<td>A-11029</td>
<td>1/1000</td>
</tr>
<tr>
<td>Alexa Fluor® 555 goat anti-mouse IgM (µ chain)</td>
<td>A-21426</td>
<td>1/1000</td>
</tr>
</tbody>
</table>
**Determination of Saturation in Co-Xgal/Immunofluoresence Stained Cultures**

Intensity of *RbP-LacZ* activity was determined by measuring RGB (Red-Green-Blue) levels of cells which showed Xgal positivity as determined by presence of blue precipitate in bright field images. RGB levels were measured using ImageJ from the NIH and then converted to saturation levels by converting RGB data to HSV (Hue-Saturation-Value) data using models developed for graphics analysis (Agoston, 2005). The hue of all cells is designated as blue. Saturation measures the departure from white and is defined as the (Max (RGB) value − Min (RGB) value) / Max (RGB) value. Since the blue hue was held constant, the calculation becomes (Blue(RGB)− Min(RGB)) / Blue(RGB). This was confirmed as all cells had Blue (RGB) as the max value. Saturation distribution plots were generated by sorting saturation values into bins and the frequency of occurrence of each bin was plotted on the y-axis.

**Measurement of Nuclear Area in Primary Neuron Culture**

Nuclear area was calculated from 40x DAPI images of either Day 6 images taken after combined Xgal and immunofluorescent experiments or from Day 4 single immunofluorescent experiments. DAPI stained nuclei were selected using the NIH ImageJ program and the total amount of pixels within each nucleus was measured using the measure function. Area counts were taken from at least three or more separate images. Determination of Xgal status and antibody status was confirmed by overlaying light images and fluorescent images.
REFERENCES


Kim, K. K., Adelstein, R. S., & Kawamoto, S. (2009). Identification of Neuronal Nuclei (NeuN) as Fox-3, a New Member of the Fox-1 Gene Family of Splicing Factors, 284(45), 31052-31061.


doi:10.1002/jcp.1135
Appendix

Quantification of Rb family member binding at the Rb Promoter using quantitative RT-PCR

We analyzed chromatin derived from ChIP in wild type C57BL/6 cortex at E14.5 using quantitative RT-PCR and found enrichment of RB family members at the Rb promoter that was absent at Rb intron 3 (Figure A-1). Protocols for amplification between quantitative RT-PCR and PCR were modified due to difficulty in obtaining amplification in quantitative RT-PCR. Amplification was performed using Qiagen QuantiTect SYBR Green RT-PCR Kit (Qiagen 204141) at 60°C for 40 cycles. Input samples at 1:5, 1:25, and 1:125 were run concomitantly to ensure that the amplification was linear. We saw differences in results between conventional and quantitative RT-PCR methods and this was most likely due to our inability of using Q solution in quantitative RT-PCR, which was essential for amplification of the highly GC rich region in conventional PCR. Additionally, melting curves showed potential secondary peaks, particularly for the Rb Intron 3 amplification, which indicated possible secondary product formation.
Figure A-1: Quantification of ChIP of Rb family members at the Rb promoter.

Quantification of DNA from ChIP at the Rb promoter and Rb Intron 3 shown in figure 3-2 was performed using quantitative RT-PCR. We found enrichment of Rb family members at the Rb promoter as compared to IgG and we saw no similar enrichment at Rb Intron 3 (A). Calculations for enrichment at the Rb promoter are shown (B). Briefly, the C_{T} values of the input sample (C_{T input}) were subtracted from the C_{T} of the samples to derive ΔC_{T}. The difference between the ΔCT of samples and the ΔC_{T} of the IgG were then used to determine the fold increase over Igg (2^{ΔΔC_{T}}), where levels of IgG are designated 1.0. Fragmentation of DNA by sonication showed that all DNA was between 200 and 100 bp (C).
Figure A-2: Increase in the intensity of Pax6 staining in Rb deficient cortical culture on Day 4

Immunocytochemical analysis of primary cortical cultures from E14.5 wild type (RbLoxP/+) and Rb mutant (NestinCre;RbLoxP/LoxP) cortices was performed with a monoclonal antibody against Pax6. There was an increase in the intensity, as well as a decrease in frequency, of Pax6+ cells with Rb deficiency (H) as compared to a litter mate control (F).