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STUDY OF REST AS A NEGATIVE REGULATOR OF P16INK4A

Monica B. Gireud

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STUDY OF REST AS A NEGATIVE REGULATOR OF P16^{INK4A}

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STUDY OF REST AS A NEGATIVE REGULATOR OF P16^{INK4A}

A

Thesis

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MASTERS of SCIENCE

by

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Houston, Texas

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Dedication

To God, who has blessed me with a wonderful family and amazing opportunities.

To my family who has shown me unconditional love and support. You have been my stability, my rock, and my biggest fans. You have been there every step of the way and have picked me back up when I felt like giving up. You have shown me that together, we are unbreakable.

To my parents who have been my strongest supporters, both emotionally and financially. You have guided me, supported me, and respected me as I created a path for myself. You have instilled in me a hard work ethic and the importance of a good education. You have taught me never to settle and have shaped me into the strong, independent woman I am today. Your encouragement, love, and strength are my inspiration.

To my mom, the strongest woman I know. You carry the weight of our family on your shoulders and your strength has always been the bond that keeps our family together. Without your dedication to us, we wouldn't be where we are.

To my dad, who has shown me that sometimes all you need is to work hard and have faith that it will be recognized. You have taught me to never give up and to fight for what I deserve and most importantly what I believe in. You have taught me that no matter the circumstances, perseverance and determination will lead you to greatness.

To my brothers, who are my role models. You give me strength and advice when I'm in need. Your love and support have made me want to be the best that I can be.

To my friends, who have been constant sources of motivation and inspiration.

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STUDY OF REST AS A NEGATIVE REGULATOR OF P16^{INK4A}

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The *RE1* Silencing Transcription Factor (REST) is a negative regulator of neuronal differentiation. It is expressed ubiquitously in early embryos, but downregulated in neural progenitors concomitant with onset of neuronal differentiation in these cells. REST has been widely studied as a negative regulator of neuronal differentiation genes. Our recent work identified a novel role for REST in control of cell proliferation. However, the underlying molecular mechanism(s) are not known and is a focus of the current thesis project. Here, we provide evidence that REST signaling controls the expression of the cyclin-dependent kinase inhibitor, p16^{Ink4a}, a negative regulator of the cell cycle and passage through G1. We determined that REST expression in the proliferating granule progenitors of the cerebellum and its lack of expression in the differentiated neurons is reciprocally correlated with that of p16^{Ink4a}. Decline in REST levels in differentiating primary and neural stem cells immortalized with v-myc (NSC-M) granule progenitors *in vitro* was also associated with upregulation of p16^{Ink4a} expression. Conversely, constitutive human REST transgene expression in NSC-M cells (NSC-MRs) blocked p16^{Ink4a} upregulation, even under neuronal differentiation conditions. However, the lack of a consensus REST DNA binding *RE1* element in the regulatory regions of *p16^{Ink4a}* locus suggested an indirect regulation of p16^{Ink4a} by REST. Based on work from other groups that showed repression of *p16^{Ink4a}* transcription by the polycomb protein Bmi-1, and its negative regulation by *microRNA-203* (*miR-203*) and our identification of a *RE1* element in the downstream regulatory region of *miR-203*, we asked if the p16^{Ink4a} expression was

controlled by REST through a series of negative regulatory events involving *miR-203* and Bmi-1. We observed that Bmi-1 expression mirrored that of REST and inversely correlated with that of *miR-203* in the postnatal cerebellum and *in vitro* differentiated granule and NSC-M progenitors. In contrast, forced *REST* transgene expression in NSC-MR cells abrogated the decrease in Bmi-1 levels and elevation in *miR-203* expression. Significant REST binding to the *miR-203 RE1* element was also observed in NSC-M cells, indicating that REST had the potential to directly regulate *miR-203* expression. In conclusion, our studies suggest a role for REST in control of cell cycle transit in neural progenitors through negative regulation of *p16^{Ink4a}*. Further validation of these results in REST knockout mice is needed, and is ongoing.

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Abbreviations

βArr1- β-arrestin 1

β-TRCP-β-Transducing Repeat-Containing Protein

Bmi-1- B-lymphoma Moloney Leukemia Insertion Virus Region

Bmp- Bone Morphogenic Protein

CDK- Cyclin Dependent Kinase

CDKI- Cyclin Dependent Kinase Inhibitors

ChIP- Chromatin Immunoprecipitation Assay

CPCs- Granule Cerebellar Progenitor Cells

DAPI- 4'6-diamidino-2-phenylindole

DBD- DNA Binding Domain

DMEM- Dulbecco's Modified Eagle's Medium

E- Embryonic Day

EGL- External Granular Layer

ESC- Embryonic Stem Cells

FBS-Fetal Bovine Serum

HDACs- Histone Deacetylase

HS- Heat Inactivated Horse Serum

IFA- Immunofluorescence Analysis

IgG- Immunoglobulin

IGL- Internal Granular Layer

INK4a- Inhibitor of Kinase 4

MB- Medulloblastoma

Min- Minutes

miR- MicroRNA

ML- Molecular Layer

mRNA- Messenger RNA

NLS- Nuclear Localization Signal

NRSF- Neuron-Restrictive Silencer Factor

NSC- Neural Stem Cells

NSC-M- v-Myc Immortalized Mouse Neural Stem Cells

NSC-MR- v-Myc Immortalized Mouse Neural Stem Cells Engineering to Constitutively Express a Human REST Transgene

P- Postnatal Day

p16^{Ink4a}- Cyclin Dependent Kinase Inhibitor 2a

PTCH- Patched

qRT-PCR- Quantitative Reverse Transcriptase Polymerase Chain Reaction

qPCR- Quantitative Polymerase Chain Reaction

RARE- Retinoic Acid Receptor Element

RB- Retinoblastoma

RE1- Repressor Element 1

REST - Repressor Element (RE)-1 Silencing Transcription Factor

RNU6B- U6 Small Nuclear RNA

SHH- Sonic Hedgehog

Smo- Smoothed

Syn1- Synapsin1

SVZ- Subventricular Zone

Tuj1- Neuron-Specific Class III β -Tubulin

UTR- Untranslated Region

WB- Western Blotting

Wnt- Wingless

Chapter 1
Introduction

Control of Proliferation and Differentiation in the Cerebellum.

The cerebellum is the region of the brain that is important for coordinating motion and balance, motor learning, and higher cognitive functions. The cerebellum develops from the posterior neural tube (1, 2). Although it composes only about 10% of total brain volume, it contains more than half of the neurons in the brain (1, 2). In humans, the cellular organization of the cerebellum is in flux for approximately one year after birth (1), making it highly vulnerable to developmental irregularities, such as medulloblastoma (MB), a highly malignant pediatric brain tumor.

The cerebellum is derived from cells in the ventricular zone and the rhombic lip (1, 2). The subventricular zone (SVZ) is comprised of the Purkinje cells of the cerebellar cortex and the Bergman glia cells (1, 2). The rhombic lip gives rise to the granular neural precursors, or cerebellar progenitor cells (CPCs) (Fig. 1A and 1B) (1, 2). In mice, the Purkinje cells develop around embryonic day (E) 13, and, shortly thereafter, migrate radially over the already formed deep cerebellar nuclei (Fig. 1B). There they settle beneath the external granular layer (EGL), awaiting the inward migration of the CPCs (1). At approximately E11, CPCs begin their first round of proliferation in the rhombic lip and initiate their migration over the surface of the cerebellum to form the outer EGL at approximately E27 (1). These cells display the proliferation markers *Math1* and *Nestin* (Fig. 1A) (1, 4, 5). The CPCs in the outer EGL undergo their second round of proliferation shortly after birth (1).

There are four major pathways that control CPC expansion, the Sonic hedgehog (SHH) pathway, the Notch pathway, the Wingless (Wnt) pathway, and the Bone Morphogenic Protein (Bmp) negative growth regulator pathway (6-9). SHH was the first

key mitogen discovered to promote CPC neurogenesis (6) and its role in neurogenesis has been extensively studied. For CPC proliferation to occur post-natally, the Purkinje cells secrete SHH (1, 2, 10). SHH ligand binds to the Patched (PTCH) receptor found on the membrane of CPCs in the outer EGL (7, 10) (Fig. 1C). In the absence of SHH, PTCH1 binds to Smoothed (Smo), a G-protein coupled receptor necessary for SHH-mediated signaling. Its association with PTCH prevents its translocation into the nucleus. Thus, when SHH is present, PTCH1 dissociates from Smo, and the latter then signals the CPCs in the EGL to expand and proliferate (7, 10).

Eventually, CPCs switch from a proliferation stage to a differentiating stage through cell-intrinsic mechanisms. The SHH mitogenic pathway contains a negative feedback loop that drives CPC cell cycle exit. In the presence of SHH, β -arrestin 1 (β Arr1), a negative regulator of G-protein coupled receptors such as Smo, begins to accumulate (11). Subsequently, β Arr1 is phosphorylated at serine 412, causing a translocation from the cytoplasm to the nucleus where it participates with CREB and p300 to enhance expression of cyclin dependent kinase inhibitor p27^{Kip1} (11). Accumulation of p27^{Kip1} ultimately drives CPC cell cycle exit, causing the CPCs to begin migrating downward, forming post-mitotic neurons within the inner EGL (Fig. 1D). These post-mitotic neurons express early neuronal differentiation markers such as p27^{Kip1}, NeuroD and neuron-specific class III β -tubulin (Tuj1) (1, 10, 12). They subsequently undergo terminal differentiation, extending axons that form synapses with Purkinje cells as they continue to migrate through the molecular layer to form mature neurons in the IGL (Fig. 1D) (10). The neurons in the IGL express neuronal differentiation markers such as Synapsin1 and Synaptophysin.

In human cerebellar development, most cells in the IGL differentiate into neurons after the first year concomitant with disappearance of the EGL. However, since the cerebellum takes approximately a year to fully develop, it is highly susceptible to developmental irregularities. Mutations in the genes of the SHH pathway have been linked to excessive proliferation and account for about 30% of sporadic MB tumors (Fig. 1E) (1). A balance between positive and negative regulators of neurogenesis prevents these irregularities from occurring. In 1995, two groups independently identified a gene known as the Repressor element 1 (*RE1*) Silencing Transcription Factor (*REST*)/Neuron-Restrictive Silencer Factor (*NRSF*), a negative regulator of neuronal phenotype (13, 14). Subsequent studies identified a critical role for REST as a negative regulator of neuronal genes in embryonic stem cells (ESC), neural stem cells (NSC)/progenitor cells, and non-neural cells (15-19).

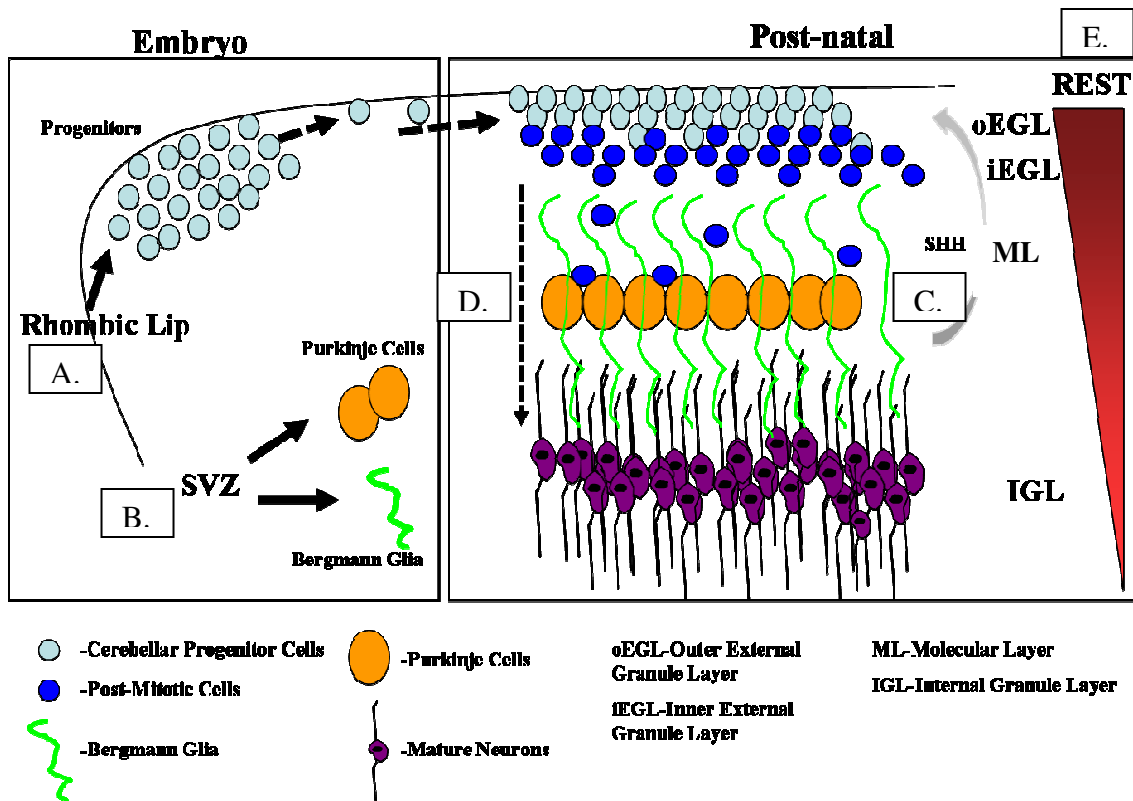
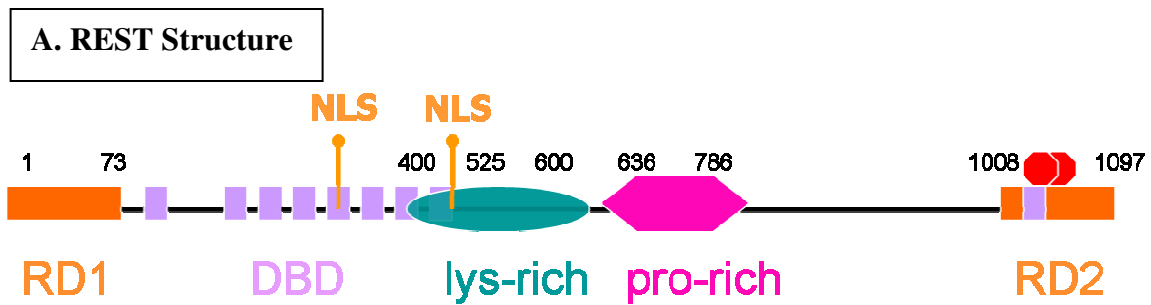


Figure 1: Cerebellar Development. A.) and B.): The rhombic lip gives rise to the cerebellar progenitor cells (CPCs) that migrate toward the outer external granular layer (EGL) while the ventricular zone gives rise to the Purkinje neurons and Bergmann glia that settle beneath the EGL. C.) The Purkinje cells secrete a mitogen, Sonic hedgehog, (SHH) that signals the CPCs in the EGL to expand and proliferate. D.) Cessation of SHH signaling promotes CPC migration to the inner EGL to form post-mitotic neurons. These cells then differentiate and continue to migrate through the molecular layer and become terminally differentiated neurons in the internal granular layer (IGL) (Fig. 1D). E.) Repressor element 1 (*RE1*) Silencing Transcription Factor (*REST*) is a major regulator of cerebellum development (13, 14). (Adapted from Dahmane and Altaba, 1999 and Wang and Zoghbi, 2001)

REST is a Repressor of Neuronal Differentiation Genes

REST is a transcriptional repressor of neuronal differentiation genes (14). It is found in human, mouse, chick, fugu, and many other organisms (19, 20). In mice, *REST* expression is highest in non-neural cells of the developing embryo, where neuronal genes must be silenced (17). Studies of *REST* null mice showed they have malformed brains, exhibit ectopic expression of some neuronal differentiation genes and die early in development at E11.5. (19). *REST* is also expressed in ESCs, NSCs, and non-neural cells, but is downregulated in most neurons, consistent with its role as a negative regulator of neuronal genes (13, 14, 19, 21, 37). This suggests REST may be important for brain development in vertebrates.

REST is a 116 kDa zinc finger protein that contains a DNA binding domain localized within a central cluster of eight zinc finger domains (Fig. 2A) (13, 15). REST has a proline-rich region and a lysine-rich region that contains one of its nuclear localization signals (NLS) (Fig. 2A) (13, 15). The other NLS is found on zinc finger 5 (13, 15). It also has two separate repressor domains located at the amino-(N) and carboxy-(C) terminus (Fig. 2A) (13, 15). The N-terminal repressor domain interacts with mSin3A while the C-terminal repressor domain interacts with CoREST (15, 22). Both repressor domains recruit histone deacetylase (HDACs) silencing complexes to remodel chromatin and repress gene expression (15, 22). In addition, REST contains two degron sequences in the c-terminus (23, 24).



B. *RE1* Consensus Sequence

5'-TTCAGCACCNCGGACAGNGCC-3'

Figure 2: Structure of REST protein and its DNA binding Consensus Sequence.

A.) REST contains a DNA binding domain (DBD) localized within a cluster of eight zinc finger domains. It has two nuclear localization signals (NLS), one on zinc finger 5 and the other in the Lys-rich sequence. It also contains two degron sequences in the c termini and two separate repressor domains located at the amino-(N) (RD1) and carboxy-(C) (RD2) termini of the protein. B.) REST binds to the *RE1* consensus sequence in the regulatory regions of its target genes. (Adapted from Chong et al., 1995; Schoenherr et al., 1995; and Ballas et al., 2005)

A number of studies have determined that REST binds to a 21-23 base pair consensus DNA binding motif called the *REI* element in the regulatory region of its target genes (Fig. 2B) (13, 14, 20, 21). These sites may be proximal or distal to the transcription start site, or they may be located within introns (20). Recent genome-wide analysis of potential *REI* sites revealed that there are over 1,892 human, 1894 mouse, and 554 Fugu *REI* sites identified as potential target genes of REST (20). Many of these genes are expressed within the nervous system and known to encode essential neuronal traits such as ion channels, synaptic vesicle proteins, vesicular trafficking/fusion, and neurotransmitter receptors (20, 25). Due to the number of *REI* sites, REST target genes are divided into three classes. Target genes that are directly upregulated in the absence of REST are known as class I genes (17, 19). Class II REST target genes require both the absence of REST function as well as the presence of other positive activators (17, 19). There is a new emerging class of REST target genes, Class III, that require both the absence of REST as well as relief from other repressive activities, such as CoREST (21).

Interestingly, the genome-wide sequence also revealed that some short-noncoding RNA, known as microRNAs (miRs), have *REI* binding sites. MiRs regulate gene expression at the post-transcriptional level by binding to the 3' untranslated regions (3'UTR) of their target messenger RNA (*mRNAs*) and silence gene expression by either degrading the *mRNA*, or reducing translation efficiency during tissue development (26-30). Specifically, REST regulates expression of many miRs known to promote neuronal differentiation such as *miR-124a*, *miR-9*, and *miR-132* (22). One of the first miRs shown to be regulated by REST was *miR-124a*, which is known to repress non-neuronal transcripts. Thus, in non-neural cells, REST binds to *miR-124a* to repress its expression

and promote non-neuronal transcripts (31, 32). However, in neuronal cells, REST is no longer repressing *miR-124a*, causing rapid degradation of non-neuronal transcripts allowing a neuronal phenotype (31). Additionally, in mouse ESCs, REST represses expression of *miR-21*, a negative regulator of some self-renewal genes such as Sox2, c-Myc, Nanog, and Oct4 (33).

To further assess the importance of REST-mediated repression on neurogenesis, previous studies constructed a recombinant transcription factor, REST-VP16, which binds to the same DNA binding site but instead of the two repressor domains, it contains an activation domain of herpes simplex virus protein VP16 (34). This group found that REST-VP16 can compete with endogenous REST for binding and transcriptionally activate REST target genes (35, 36). Additionally, in NSCs, the direct activation of REST target genes by REST-VP16 was sufficient to induce differentiation of NSCs into functionally active neurons and convert the cells into a mature neuronal phenotype (35).

Moreover, REST can function as an activator of a subset of its own neuronal target genes in adult neurogenesis. When REST was bound to a non-coding double stranded RNA (dsRNA) with similar homology to the *RE1* site, REST functioned as an activator of its target neuronal differentiation genes in NSCs leading to neuronal differentiation (37).

REST itself is regulated at the post-translational level. In non-neural cells, β -Transducin repeat-containing protein (β -TRCP), an E3 ligase, promotes ubiquitin-mediated proteosomal degradation of REST during the G2 phase of the cell cycle (24). Similarly, when ESCs progress on their way to lineage restricted neural progenitors, REST protein is degraded by β -TRCP-mediated ubiquitylation and the proteosomal

degradation complex (23). At this point, although protein levels are being lowered by degradation, the REST-corepressor complex still occupies and represses its target genes. This repression in NSCs is weak in comparison to the repression in non-neural cells and ESCs, where neuronal genes must be permanently silenced (17). REST transcript levels do not decrease until the NSCs exit the cell cycle and differentiate into neurons. REST transcription was shown to be negatively regulated by the binding of the unliganded retinoic acid receptor element (RARE) found on the REST promoter (15). The downregulation of REST expression concomitant with neuronal lineage specification is associated with derepression of REST target genes (15, 21, 22).

REST is an Oncogene in Neural Tumors

Medulloblastoma, a malignant pediatric brain tumor, is characterized by poor neuronal differentiation and hyper-proliferation (3, 35, 38). Previous studies have shown elevated REST expression in human MB samples compared to normal age-matched cerebella (35, 38). These results suggest a defect in pathways controlling the downregulation of REST expression (21, 38). Countering REST expression in the human MB cell line, DAOY, abrogated the tumorigenic potential of these cells in mouse orthotopic models (38). Conversely, v-myc immortalized mouse cerebellar progenitor cells (NSC-Ms) engineered to constitutively express REST under a doxycycline promoter (NSC-MRs) failed to differentiate and maintained a proliferative state as shown by an immunocytochemical assay for BrdU incorporation (Fig. 3) (3). Additionally, ectopic REST expression in murine progenitors promoted formation of cerebellar tumors in murine cerebellum, implicating REST in MB formation (3) and Immunofluorescence Analysis (IFA) showed NSC-MR cells lacked differentiation

markers, indicating that they cannot become terminal neurons and offered a proliferative advantage. (3). More recently, we have shown that REST expression maintains proliferation in MB tumor cells at least in part through regulation of expression of p27^{Kip1} (73).

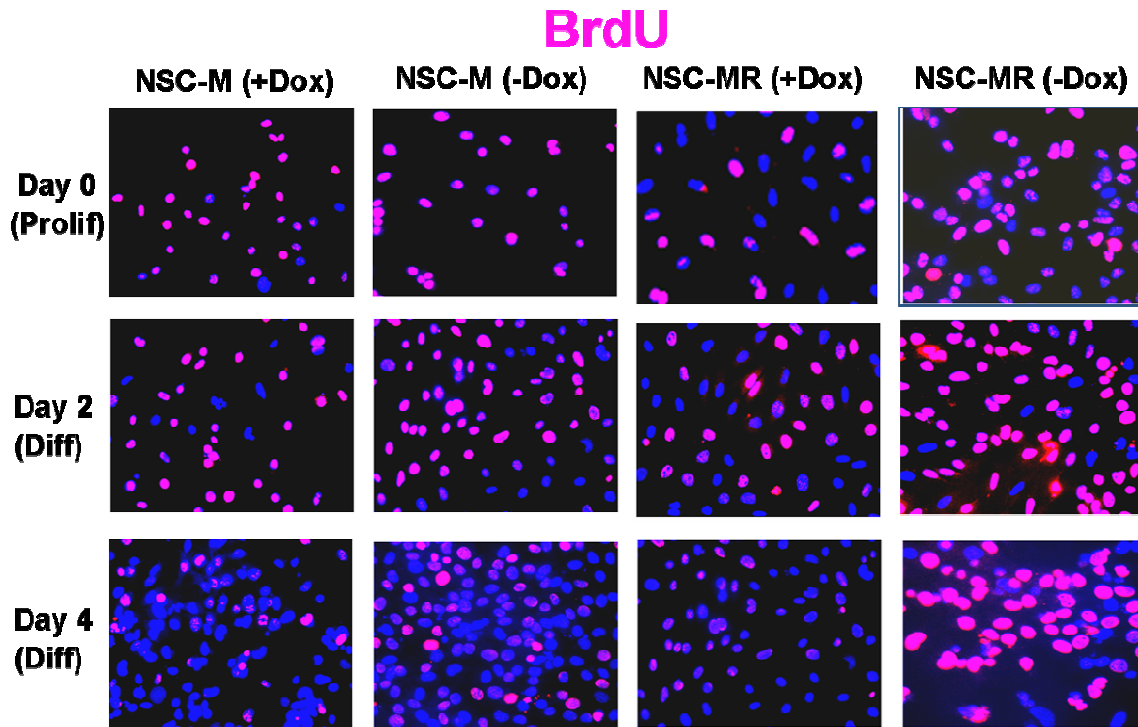


Figure 3: An immunocytochemical assay for BrdU incorporation in NSC-M and NSC-MR cells. Cells were plated under either proliferation or differentiation condition, BrdU was added to the media and the cells were washed, fixed, and stained with anti-BrdU for immunocytochemical analysis. NSC-M cells incorporate BrdU at a much lower rate under differentiation conditions compared to proliferation conditions. In contrast, NSC-MR cells expressing the human REST transgene (NSC-MRs -Dox) incorporate BrdU at high rates even when cultured under differentiation conditions (3).

REST is a Tumor Suppressor in Non-Neural Tumors

A variety of human tumors arising from epithelial cells of tissues including breast, ovarian, colorectal, and lung cancer, show abnormal activation of neuron-specific genes. This suggested a defect in the regulators of neuronal genes. REST is normally widely expressed throughout non-neural tissues to silence neuronal genes. Therefore, when it is deleted or mutated, some of its neuronal genes are aberrantly expressed. REST was identified as a frequently deleted gene in colorectal cancer (39). In addition, using an RNA interference mechanism, blocking REST function in the mammary epithelial cells was shown to cause a transformation phenotype leading to a transformed breast cell (39). Thus, the inhibition of REST function seems to be a common event that occurs in diverse tissues during normal physiological responses, as well as disease.

REST Expressing Cells offer a Proliferation Advantage.

Previous studies showed that NSC-MR cells offer a proliferation advantage over NSC-M cells as shown by an immunocytochemical assay for BrdU incorporation (3) (Fig. 3). We have shown that *REST* knockdown in NSC-Ms and human MB cell lines (DAOY and D283), inhibits proliferation as shown by a reduced cell number (Fig. 4) (73). To verify if this was indeed through a proliferation mechanism, we stained for the proliferation marker Ki-67 and found a decrease in Ki-67 positive cells (Fig. 4) (73).

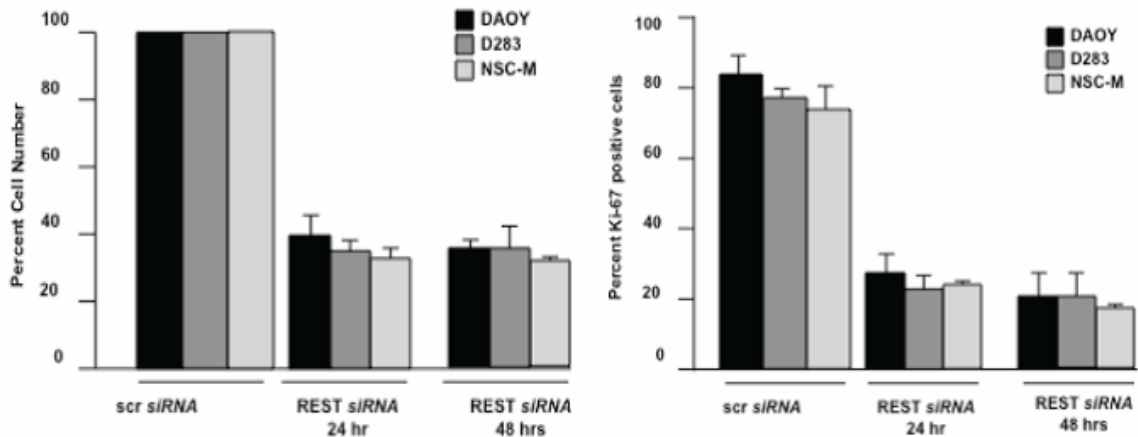


Figure 4: *REST* knockdown by transient transfection in NSC-M cells and two MB cell lines (DAOY and D283). Cells were transiently transfected with pooled *REST* specific *siRNA* or control scrambled (*scr*) *siRNA* using DharmaFect transfection reagent. *REST* knockdown promoted a decline in total cell number (left panel) and a decline in the number of Ki-67 positive cells (right panel). (73).

Together, these results implicate *REST* in controlling cell proliferation. *REST* is expressed in the G1 phase of the cell cycle and is targeted for degradation by β -TRCP during the G2 phase of the cell cycle (24). Transit through the cell cycle is governed by the activity of a family of proteins called the cyclin dependent kinases (CDKs) (Fig. 5). These CDKs interact with regulatory subunits known as cyclins and form heterodimers to phosphorylate retinoblastoma (RB) and allow progression through the cell cycle (Fig. 5) (40). The activity of these CDK/Cyclin interactions is controlled by the CDKIs, which include the kip family (p21^{Cip1} and p27^{Kip1}) and the Inhibitor of Kinase 4 family (Ink4) (p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c}) (Fig. 5) (40). CDKIs are known inhibitors of cell proliferation. Overall, these results suggest that *REST* may control cell proliferation by

negatively regulating the expression of one or more CDKIs. Our lab recently demonstrated a novel role for REST in the regulation of the cell cycle and proliferation through negative regulation of p27^{Kip1} (73). However, the pathways responsible for the other CDKIs are still relatively unknown. Interestingly, p16^{Ink4a} expression is relatively low in progenitor cells and premature expression of p16^{Ink4a} contributes to the reduced self-renewal of neural stem cells in culture (41-43). Furthermore, p16^{Ink4a} expression also coincided with a significant decline in proliferation.

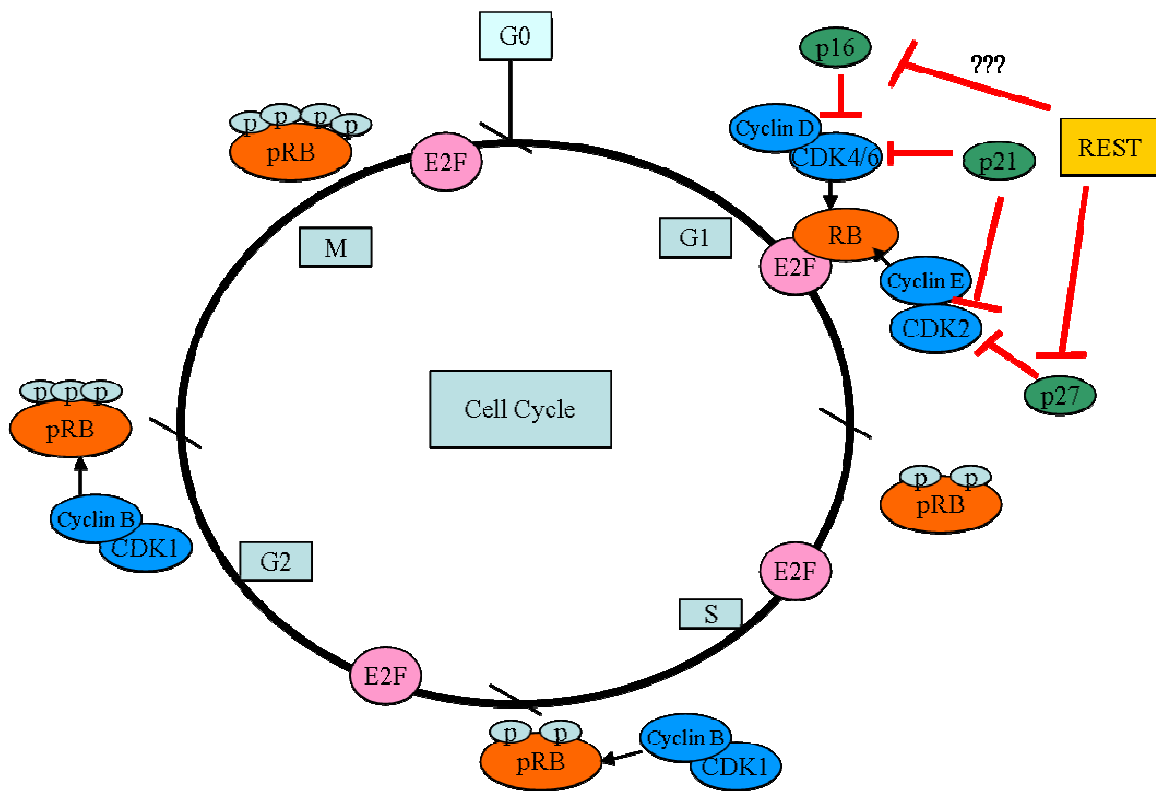


Figure 5: Progression through the cell cycle is governed by the CDK-cyclin interactions. Transit through the cell cycle is governed by CDK-cyclin interactions. Together, they form heterodimers to phosphorylate RB and allow progression through the cell cycle. These CDK/Cyclin interactions are negatively regulated by the CDKIs (40).

Hypothesis

The overall goal of my project is to elucidate the molecular mechanism(s) underlying REST-mediated control of cell proliferation during neuronal differentiation.

To summarize, we know that:

- 1.) REST overexpressing NSC-MR cells offer a proliferation advantage compared to NSC-M cells as shown by an immunocytochemical assay for BrdU incorporation (3).
- 2.) *REST* knockdown in NSC-M and human MB cell lines promotes a decrease in cell number and corresponding decrease in the proliferation marker Ki-67 (73).
- 3.) REST is expressed in the G1 phase of the cell cycle and is targeted for proteosomal degradation by β -TRCP in G2 phase of the cell cycle (24).
- 4.) Progression through the cell cycle is governed by CDK/cyclin activity. The CDKS are regulated by CDKIs (40).

The research presented in this dissertation was performed to test the hypothesis that REST promotion of proliferation may in part be through negatively regulation of expression of CDKIs. Therefore, I will study the relationship between REST and the Ink4a/ARF family member, p16^{Ink4a} and demonstrate as follows:

- 1.) REST and p16^{Ink4a} correlation
 - i. *In vivo* cerebellum
 - ii. *In vitro* in NSC-M and NSC-MR cell lines
- 2.) Mechanism by which REST regulates p16^{Ink4a}

Chapter 2

REST and p16^{Ink4a} expression are reciprocally correlated during neural development

Rationale

Previous work showed that NSC-MR cells constitutively expressing a human REST transgene (NSC-MR) failed to differentiate and continued to proliferate as determined by BrdU labeling assays (3). We showed that *REST* knockdown in NSC-M cells promoted a decrease in cell numbers and a corresponding decrease in the proliferation marker Ki-67 (73). REST is expressed in the G1 phase of the cell cycle and is targeted for degradation by β -TRCP during the G2 phase of the cell cycle (24). Progression through the cell cycle is governed by cyclin/CDK activity, which is regulated by the CDKIs (40). CDKIs are known to be negative regulators of cell proliferation (40). Based on these findings we postulate that REST-mediated control of cell proliferation may in part involve negative regulation of CDKI expression or activity. Premature expression of p16^{Ink4a} was shown to cause significant decline in proliferation and reduced self-renewal of neural stem cells in culture (41-43).

In this section, we investigated the relationship between REST and the CDKI, p16^{Ink4a}, during neurogenesis in differentiating progenitors *in vitro* and in differentiating CPCs of the postnatal cerebellum. We show that REST and p16^{Ink4a} levels are reciprocally correlated in the developing mouse cerebellum at postnatal day 7 (P7) and P12 by immunofluorescence analysis (IFA). REST and p16^{Ink4a} expression was also studied by reverse transcriptase polymerase chain reaction (qRT-PCR), Western Blotting (WB) and IFA during neuronal differentiation of progenitors *in vitro*. We observed that REST *mRNA* and protein levels in NSC-M cells were expressed in progenitors whereas p16^{Ink4a} was not. Conversely, REST was downregulated in neurons whereas p16^{Ink4a} was

detected. Furthermore, constitutive REST expression in NSC-MR cells blocked upregulation of p16^{Ink4a} *mRNA* and protein levels, even when cultured under differentiation conditions. Together, our data suggests a reciprocal relationship between REST and p16^{Ink4a}.

Results

REST and p16^{Ink4a} are reciprocally expressed in the developing post-natal cerebellum of mice.

In order to determine if REST reciprocally correlated with p16^{Ink4a} *in vivo*, REST and p16^{Ink4a} protein was analyzed in the developing cerebellum of P7 and P12 mice. C57BL6 mice were mated and the brains of the resulting pups were harvested using IACUC approved protocols various days after birth and analyzed by IFA assays using commercially available anti-REST (red) or anti-p16^{Ink4a} (green) antibodies. In P7 mice, REST is mostly in the proliferating cells of the EGL where cells are undergoing maximal proliferation while p16^{Ink4a} is in the post-mitotic and terminally differentiated neurons of the ML and IGL (Fig. 6). In P12 mice, REST levels are declining as the EGL is diminished to a few layers of progenitor cells (Fig. 6). Most of the cells are now expressing p16^{Ink4a} and are post-mitotic and terminally differentiated neurons (Fig. 6).

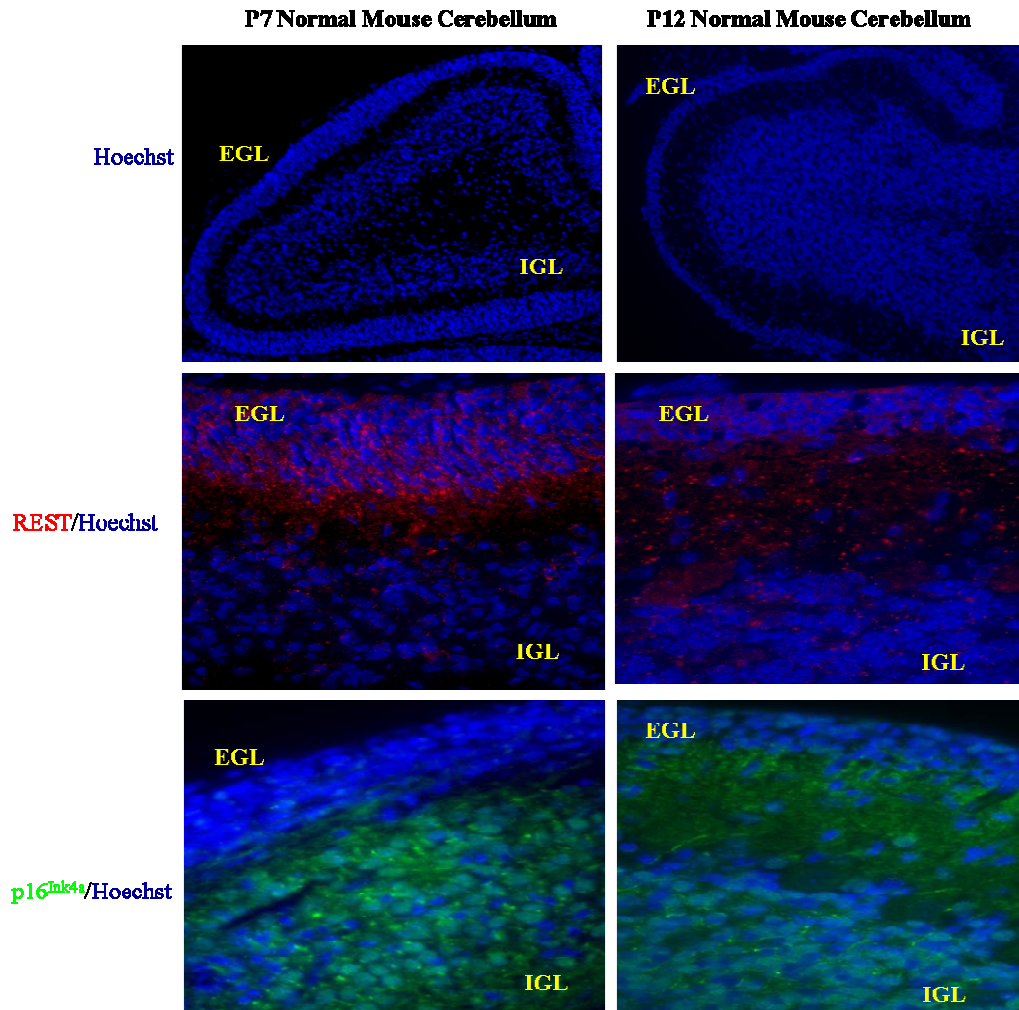


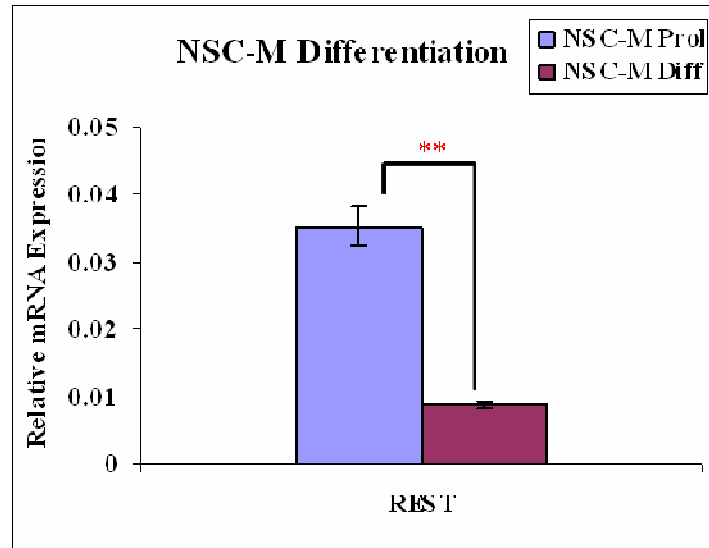
Figure 6: REST and p16^{Ink4a} are reciprocally expressed in the developing post-natal cerebellum of mice. C57BL6 wild-type mice were mated and the brains of the resulting pups were harvested using IACUC approved protocols. REST and p16^{Ink4a} protein in normal P7 and P12 mouse cerebellum was measured by immunofluorescence assay using commercially available anti-REST (red) and anti-p16^{Ink4a} (green) antibodies. Nuclei were stained with Hoechst dye and representative images were captured using a 10X lens (top panel) or 40X oil immersion lens (middle and bottom panel) on a Nikon fluorescence microscope. Images were analyzed with Metamorph software. Experiments were performed in duplicate. EGL-External Granule Layer; IGL- Internal Granule Layer.

REST expressing cells blocked p16^{Ink4a} upregulation.

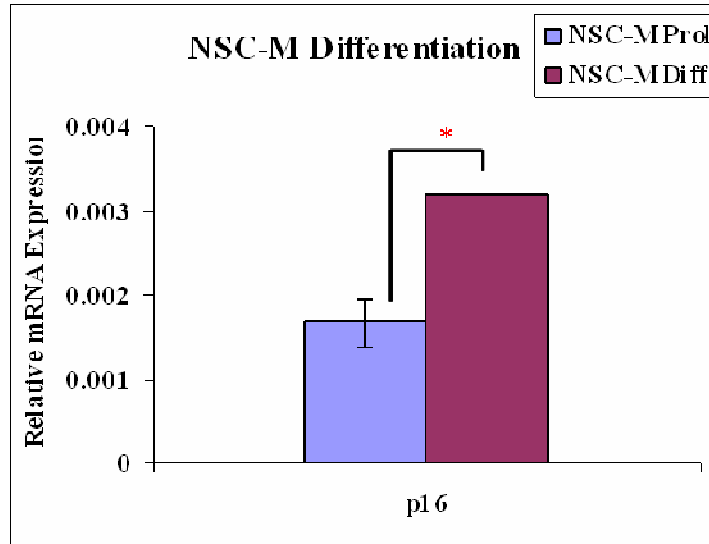
To examine the relationship between REST and p16^{Ink4a} during neuronal differentiation of neural progenitors *in vitro*, NSC-M cells were cultured under proliferation or differentiation conditions. Total RNA was collected and *mRNA* levels were quantified by qRT-PCR analyses. Relative *REST* and *p16^{Ink4a}* *mRNA* levels were determined following normalization to *18S mRNA* levels. Downregulation of *REST* gene expression during neuronal differentiation significantly correlated with upregulation of *p16^{Ink4a}* gene expression (Fig. 7). The terminal differentiation marker Synapsin1 (Syn1) was used as a positive control since it is an established REST target gene.

Conversely, NSC-MR cells cultured under these conditions maintained *REST* expression and failed to upregulate *p16^{Ink4a}* *mRNA* levels, even when cultured under neuronal differentiation conditions (Fig. 7). Together these findings suggest that maintenance of REST expression and a failure to upregulate p16^{Ink4a} expression may contribute to uncontrolled proliferation of NSC-MR cells.

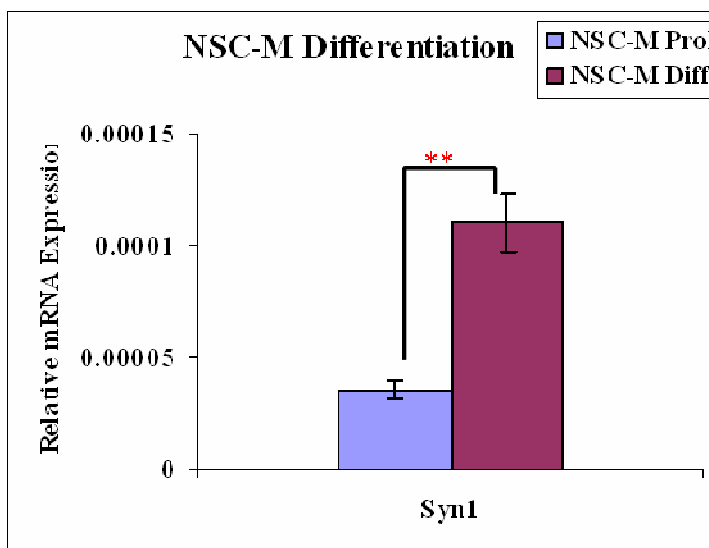
A.



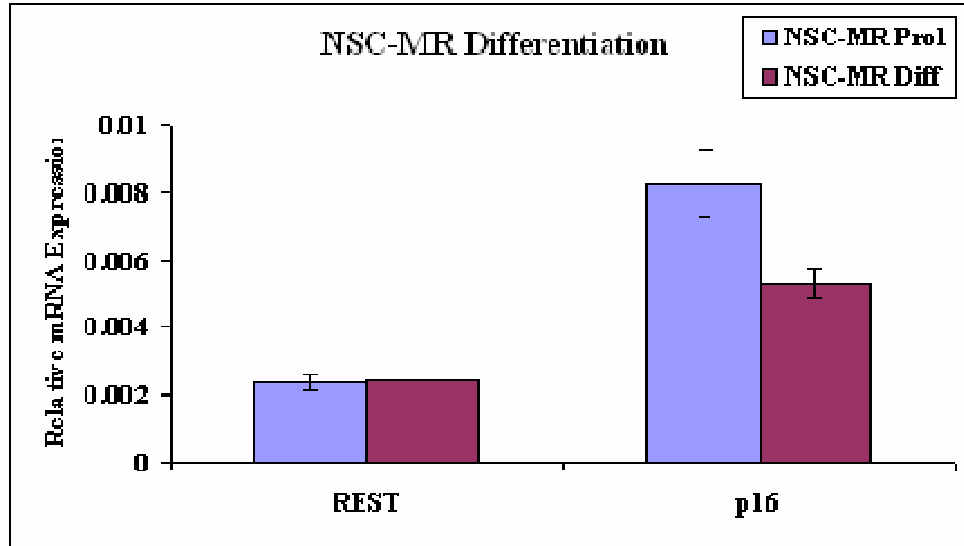
B.



C.



D.



E.

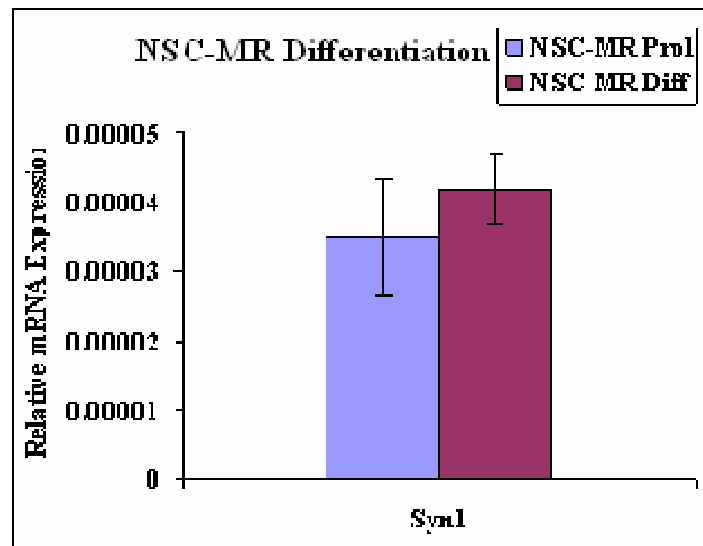


Figure 7: Maintenance of REST expression blocks $p16^{Ink4a}$ upregulation. NSC-M (A, B, and C) and NSC-MR (D, and E) cells were grown under proliferation or differentiation conditions. Total RNA was prepared, converted to cDNA and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *REST*, $p16^{Ink4a}$, and *Synapsin1* (Syn1). Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

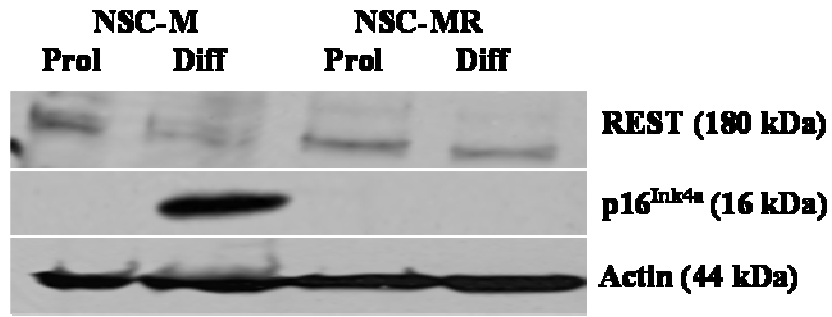
Lowered p16^{Ink4a} protein levels in Neural Progenitors with Constitutive REST expression

To further validate the reciprocal relationship between REST and p16^{Ink4a} during neuronal differentiation of neural progenitors, we analyzed protein levels by WB. NSC-M and NSC-MR cells were cultured under proliferation or differentiation conditions and whole cell extracts were analyzed by WB and probed with commercially available anti-REST or anti-p16^{Ink4a} antibodies (Fig. 8A). Actin was used as a loading control. In NSC-M cells, REST levels decline upon neuronal differentiation while p16^{Ink4a} levels are upregulated (Fig. 8A). In contrast, REST levels do not change in NSC-MR cells cultured under differentiation conditions and these cells failed to upregulate p16^{Ink4a} levels (Fig. 8A).

REST and p16^{Ink4a} protein levels and sub-cellular localization were also studied by IFA. NSC-M and NSC-MR cells were cultured in CC2 chamber slides under either proliferation or differentiation conditions, fixed and stained with anti-REST (red) or anti-p16^{Ink4a} antibodies (green). 4'6-diamidino-2-phenylindole (DAPI) (blue) was used to stain the nuclei. In NSC-M cells, REST was detected in both the nucleus and cytoplasm of progenitor cells but was only detected in the cytoplasm of differentiated neurons (Fig. 8B). In contrast, p16^{Ink4a} was not expressed in progenitor cells and was only expressed in the nucleus of differentiated neurons (Fig. 8B). Thus, downregulation of REST protein levels during neuronal differentiation in NSC-M cells correlated with upregulation of p16^{Ink4a} protein levels (Fig. 8B). Conversely, NSC-MR cells maintained REST levels and blocked upregulation of p16^{Ink4a} protein levels, even when cultured

under neuronal differentiation conditions (Fig. 8B), thereby confirming our findings in Fig. 7 and Fig. 8A.

A.



B.

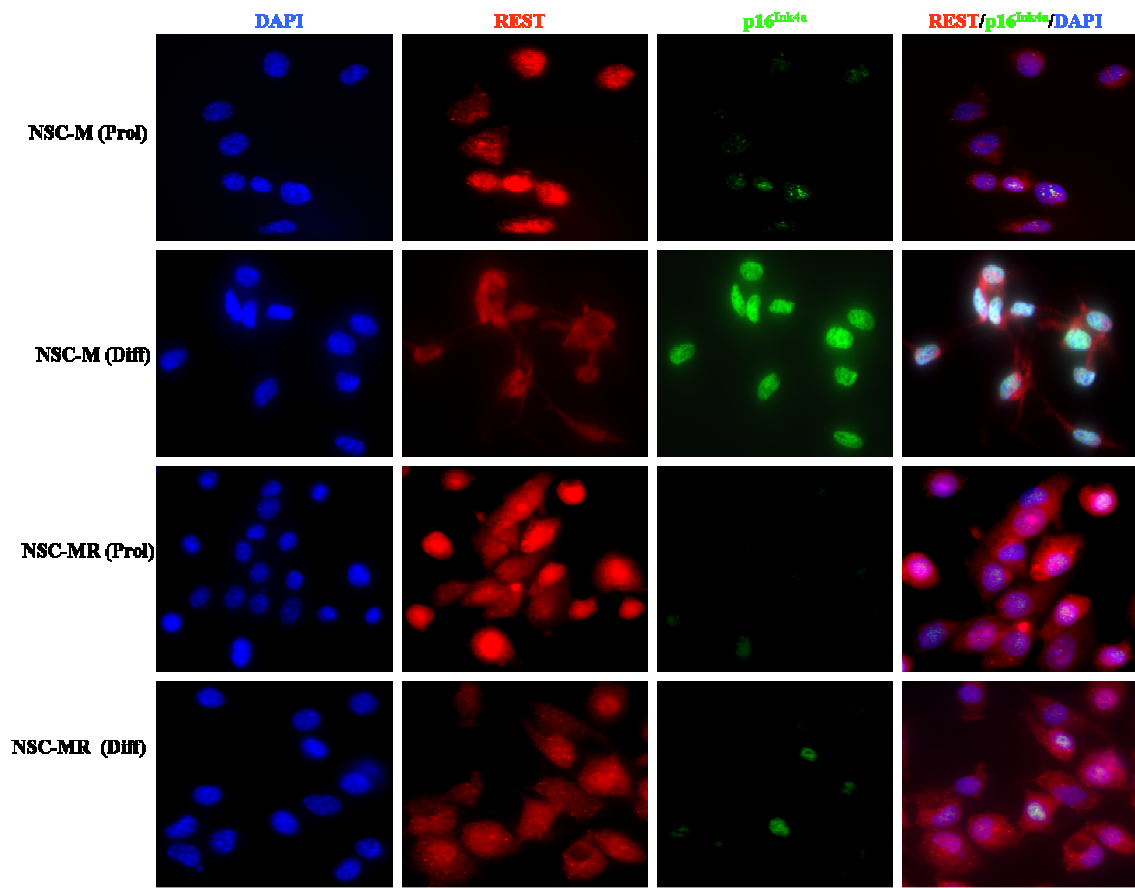


Figure 8: Maintenance of REST levels blocks p16^{Ink4a} upregulation. NSC-M and NSC-MR cells were cultured under proliferation or differentiation conditions. A.) Whole cell extracts were analyzed by Western blotting to measure changes in REST and p16^{Ink4a} protein levels. Actin was used as a loading control. Experiments were done in triplicate. Actin was used as a loading control. Experiments were done in triplicate. B.) Cells were processed by IFA to detect changes in protein levels and sub-cellular localization of REST and p16^{Ink4a} in NSC-M and NSC-MR cells. Cells were stained using commercially available anti-REST and anti-p16^{Ink4a} antibodies. Nuclei were stained with DAPI. These representative images were captured using a Nikon fluorescence microscope and analyzed with Metamorph software. Experiments were done in duplicate.

Summary

In this section, we investigated the relationship between REST and p16^{Ink4a} transcript and protein in mouse brain sections and in differentiating neural progenitors in vitro. Tissue sections were obtained and analyzed in the developing cerebellum of P7 and P12 mice for REST and p16^{Ink4a}. We found REST and p16^{Ink4a} expression in the proliferating cells of the EGL and post-mitotic/terminally differentiated neurons of the IGL respectively. We also cultured NSC-M cells under proliferation or differentiation conditions and demonstrated a decline in REST expression and increase in p16^{Ink4a} expression during neurogenesis of NSC-M cells by qRT-PCR, WB, and IFA. Conversely, constitutive REST expression in NSC-MR cells blocked upregulation of p16^{Ink4a}, even when cultured under differentiation conditions. These findings suggested a reciprocal relationship between REST and p16^{Ink4a}. The mechanism by which REST may regulate p16^{Ink4a} will be discussed in the next section.

Chapter 3

REST represses *miR-203*, a negative regulator of Bmi-1, to prevent p16^{Ink4a} expression and allow cell proliferation.

Rationale

In the previous section, we demonstrated a reciprocal relationship between REST and p16^{Ink4a} in neural progenitors *in vivo* and *in vitro*. Since REST is a transcriptional repressor, we first asked if REST directly controlled p16^{Ink4a} expression. We conducted a search of the upstream and downstream regulatory regions of p16^{Ink4a} gene for consensus *RE1* binding sites. However, the search did not reveal REST binding sites in the p16^{Ink4a} regulatory regions, suggesting that the effect of REST on p16^{Ink4a} may be indirect. Several lines of evidence have implicated the polycomb gene, B- lymphoma Moloney leukemia virus insertion region (Bmi-1), as a negative regulator of p16^{Ink4a} gene expression (41-45). Bmi-1 is essential for cerebellar development and is important for regulation of CPC proliferation (42, 43, 46, 47). Bmi-1 null mice exhibit a precocious increase in p16^{Ink4a} expression that contributed to the reduced self-renewal of neural stem cells in culture (43). It is a downstream target of the Shh pathway and aberrantly high levels of Bmi-1 are found in many cancers, including medulloblastomas (46). Other studies have shown that Bmi-1 is regulated by short non-coding regulatory RNA called microRNAs (miRs) such as *miR-15a*, *miR-16* and *miR-203* (48, 49). Importantly, REST controls the expression of miRs, specifically *miR-9*, *miR-124* and *miR-132* (32, 50, 51).

In this section, we investigated the hypothesis that REST mediated control of p16^{Ink4a} expression is through repression of miRs known to regulate Bmi-1 expression, as outlined in Fig. 9. We examined this hypothesis during neurogenesis in differentiating progenitors *in vitro* and in differentiating CPCs of the postnatal cerebellum. Additionally, we first examined in NSC-M and NSC-MR cells the expression of a panel of miRs

known to regulate Bmi-1 by TaqMan MicroRNA assay followed by qRT-PCR validation (in collaboration with Dr. George Calin's Lab). We then examined REST and Bmi-1 protein *in vivo* in the developing mouse cerebellum at P7 and P12 by IFA. We did a chromatin immunoprecipitation (ChIP) experiment to show REST binding to the *RE1* element in the downstream regulatory region of *miR-203* in NSC-M and NSC-MR cells. In our working model, we suggest that REST-mediated repression of *miR-203* maintains Bmi-1 expression and blocks p16^{Ink4a} expression (Fig. 9).

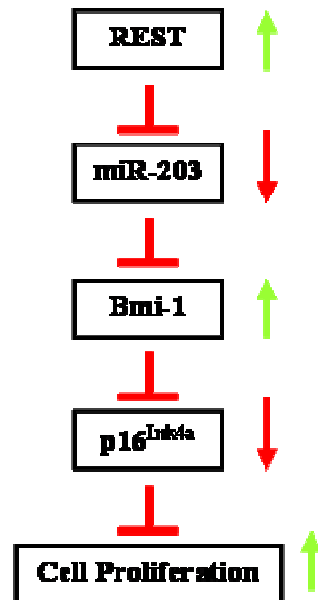


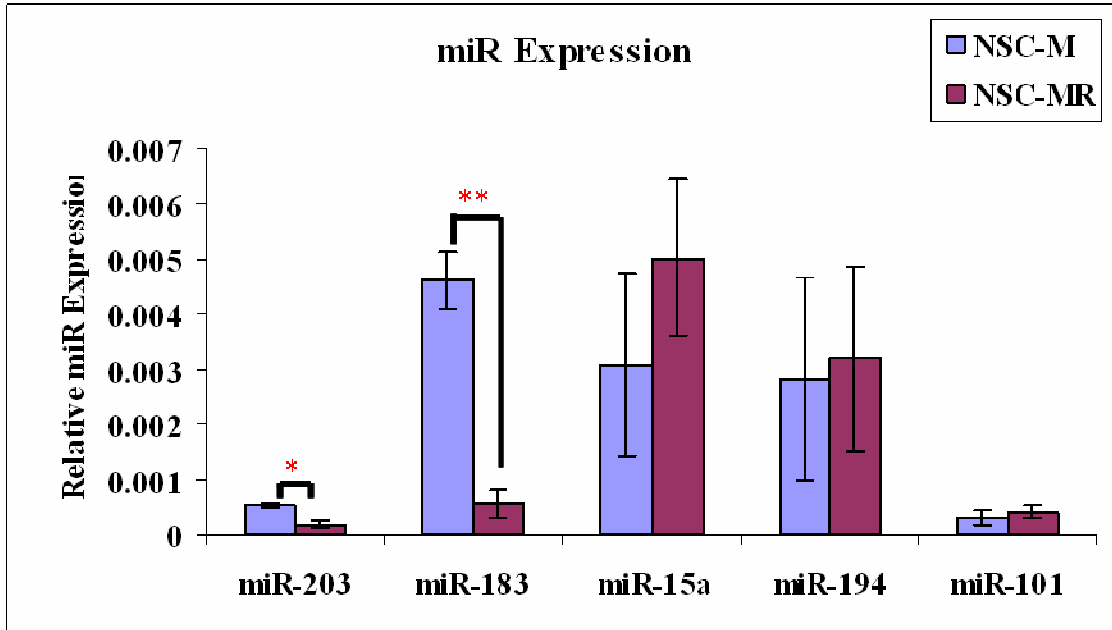
Figure 9. REST-mediated control of cell proliferation- Working Model. REST contributes to increased cell proliferation by repressing the expression of *miR-203*, which negatively regulates Bmi-1. Therefore, Bmi-1 can inactivate p16^{Ink4a}, increasing cell proliferation and allowing progression through the cell cycle.

Results

REST-dependent Changes in *miR-203* expression.

We first asked whether REST dependent negative control of p16^{Ink4a} was mediated by a miR and Bmi-1. To assess this, NSC-M and NSC-MR cells were cultured under proliferation and differentiation conditions. The expression of a panel of miRs known to control Bmi-1 were examined by TaqMan MicroRNA assay followed by qRT-PCR analyses. Gene expression was determined following normalization to U6 small nuclear RNA (RNU6B) mRNA levels. Of these, *miR-203* and *miR-183* showed a REST-dependent downregulation of expression (Fig. 10A). The expression of the other microRNAs failed to fluctuate in a REST dependent manner. Of these two miRs, only *miR-203* had a *RE1* site and therefore had the potential to be bound by REST. As highlighted in Fig. 10B, *miR-203* expression was significantly downregulated in REST overexpressing NSC-MR cells relative to NSC-M cells.

A.



B.

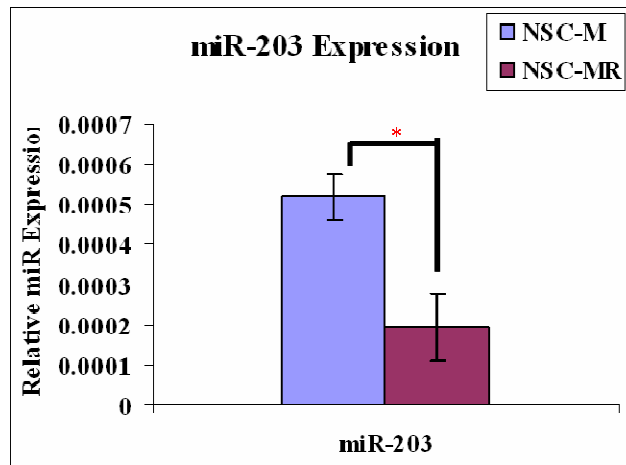


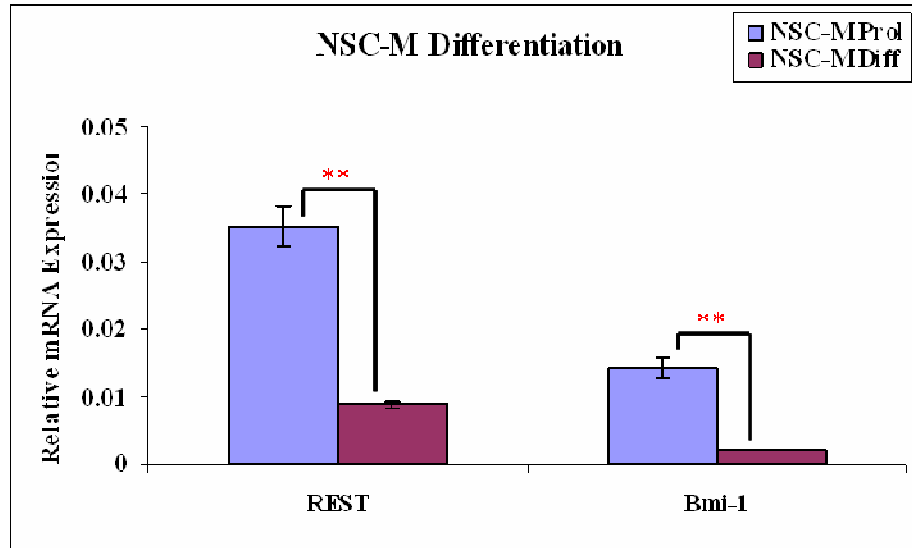
Figure 10: *Mir-203* and *miR-183* fluctuate in a REST dependent manner. NSC-M and NSC-MR cells were grown under proliferation conditions. Total RNA was prepared and miR expression was quantified by TaqMan MicroRNA assay followed by qRT-PCR analyses to measure changes in miR expression. A.) MiR expression of a panel of miRs known to regulate *Bmi-1*. B.) *Mir-203* expression is significantly downregulated in NSC-MR cells relative to NSC-M cells. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

Constitutive REST expression blocked *miR-203* upregulation and maintained Bmi-1 expression in differentiated neural progenitors.

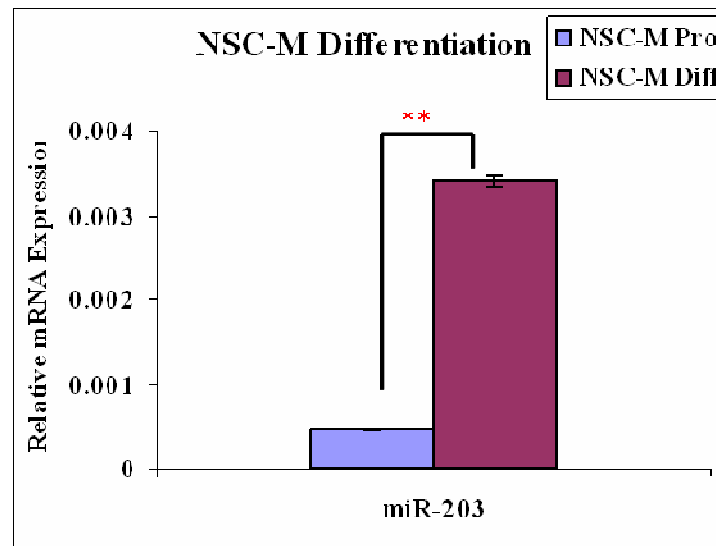
To better understand the relationship between REST, *miR-203*, and Bmi-1 expression we cultured NSC-M cells under either proliferation or differentiation conditions. Total RNA was collected, converted to cDNA and *18s*, *Bmi-1*, *Syn1*, and *REST* gene expression quantified by SYBR Green qRT-PCR. For *miR-203* measurements, total RNA was collected and *mRNA* levels were assessed by TaqMan MicroRNA assay followed by qRT-PCR analyses. *REST* gene expression (normalized to 18S expression) and *miR-203* (normalized to *RNU6B* expression) *mRNA* levels were found to be reciprocally correlated in differentiating NSC-M cells (Fig. 11). This shows downregulation of *REST* gene expression during neuronal differentiation caused a significant upregulation of *miR-203* gene expression (Fig. 11). The terminal differentiation marker Synapsin (*Syn1*), a known REST target gene was upregulated under these conditions and was used as positive control. Importantly, *Bmi-1 mRNA* levels were downregulated concomitant with a decline in *REST mRNA* levels during neuronal differentiation (Fig. 11).

In contrast, maintenance of REST expression in NSC-MR cells blocked the upregulation of *miR-203 mRNA* levels, even when cultured under differentiation conditions (Fig. 12). This correlated with maintenance of Bmi-1 *mRNA levels* in these cells (Fig. 12).

A.



B.



C.

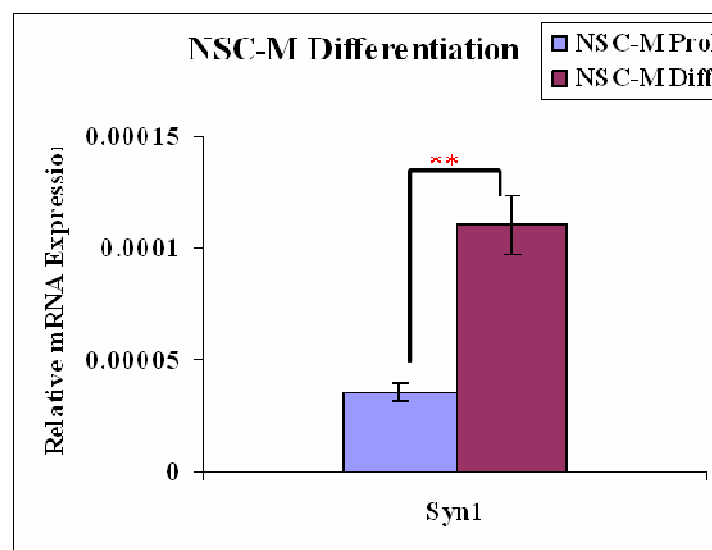
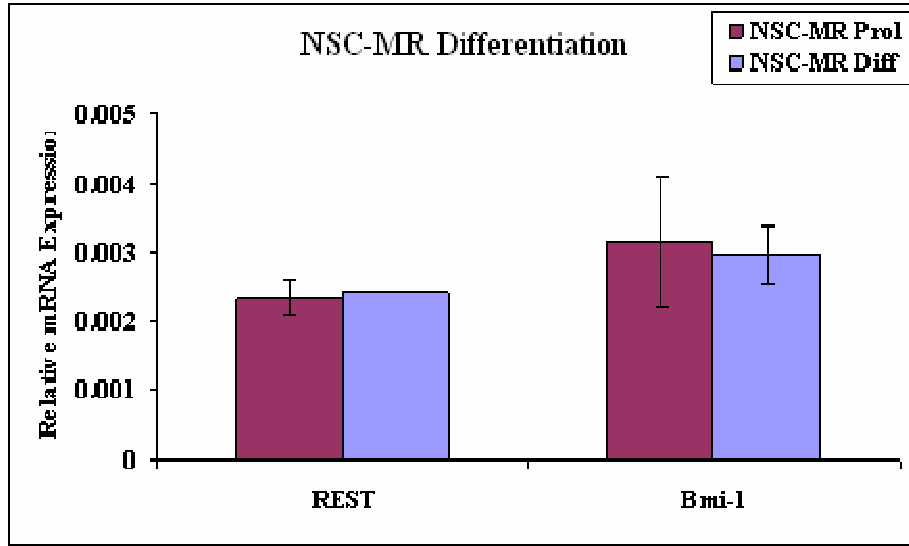
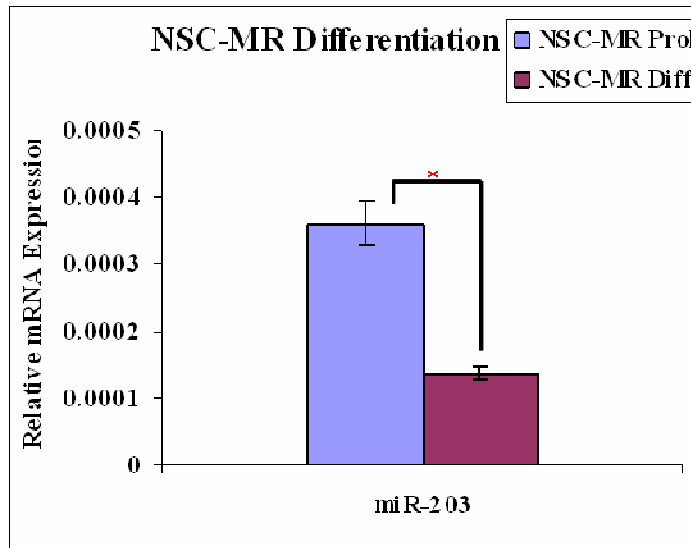


Figure 11: *REST* gene expression and *miR-203* mRNA levels were found to be reciprocally correlated in differentiating NSC-M cells. NSC-M cells were grown under proliferation or differentiation conditions. A.) Total RNA was prepared, converted to cDNA and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *REST* and *Bmi-1*. B.) Total RNA was prepared, and mRNA levels assessed by Taqman MicroRNA assay followed by qRT-PCR analyses to measure changes in gene expression of *miR-203*. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (*p<0.05, **p<0.01). C.) Total RNA was prepared, converted to cDNA and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *Synapsin1* (*Syn1*).

A.



B.



C.

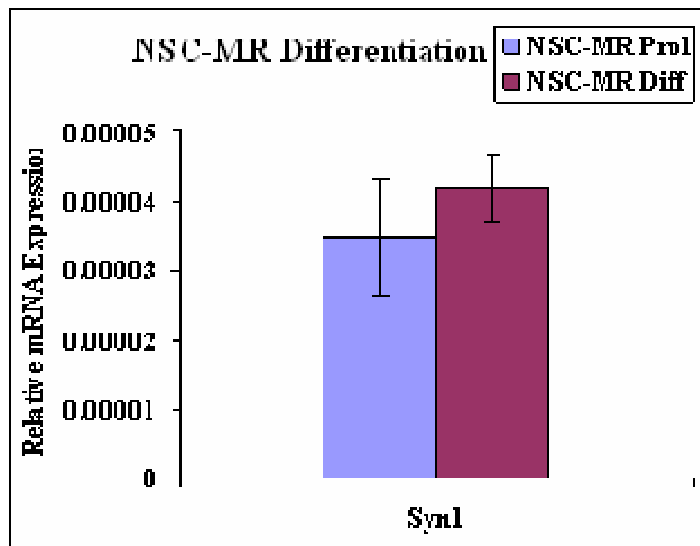


Figure 12: Maintenance of *REST* expression blocks *miR-203* upregulation and maintains *Bmi-1* expression. NSC-MR cells were grown under proliferation or differentiation conditions. Total RNA was prepared, converted to cDNA and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *REST*, *Bmi-1*, and *Synapsin1* (*Syn1*). For *miR-203*, total RNA was prepared, and *mRNA* levels assessed by Taqman MicroRNA assay followed by qRT-PCR analyses to measure changes in gene expression. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

Constitutive REST expression maintains Bmi-1 levels.

REST and Bmi-1 sub-cellular localization was also studied by IFA analyses. NSC-M and NSC-MR cells were cultured in CC2 chamber slides under either proliferation or differentiation conditions, fixed and stained with anti-REST (red) or anti-Bmi-1 (green) antibodies. DAPI (blue) was used to stain the nuclei. In NSC-M cells, REST was detected in both the nucleus and cytoplasm of progenitor NSC-M cells but was only detected in the cytoplasm of differentiated neurons (Fig. 13). Bmi-1 was found predominantly in the nucleus of the proliferating NSC-M cells and was not detected in NSC-M differentiated cells (Fig. 13). Thus, downregulation of REST protein levels during neuronal differentiation in NSC-M cells coincided with downregulation of Bmi-1 protein levels (Fig. 13). Conversely, NSC-MR cells failed to differentiate and instead maintained REST and Bmi-1 protein levels (Fig. 13). REST was detected in the nucleus and cytoplasm of NSC-MR cells under both proliferation and differentiation conditions. Bmi-1 was predominantly found in the nucleus of NSC-MR cells, even when cultured under neuronal differentiation conditions. These findings further confirmed our findings in Figs. 11, and 12.

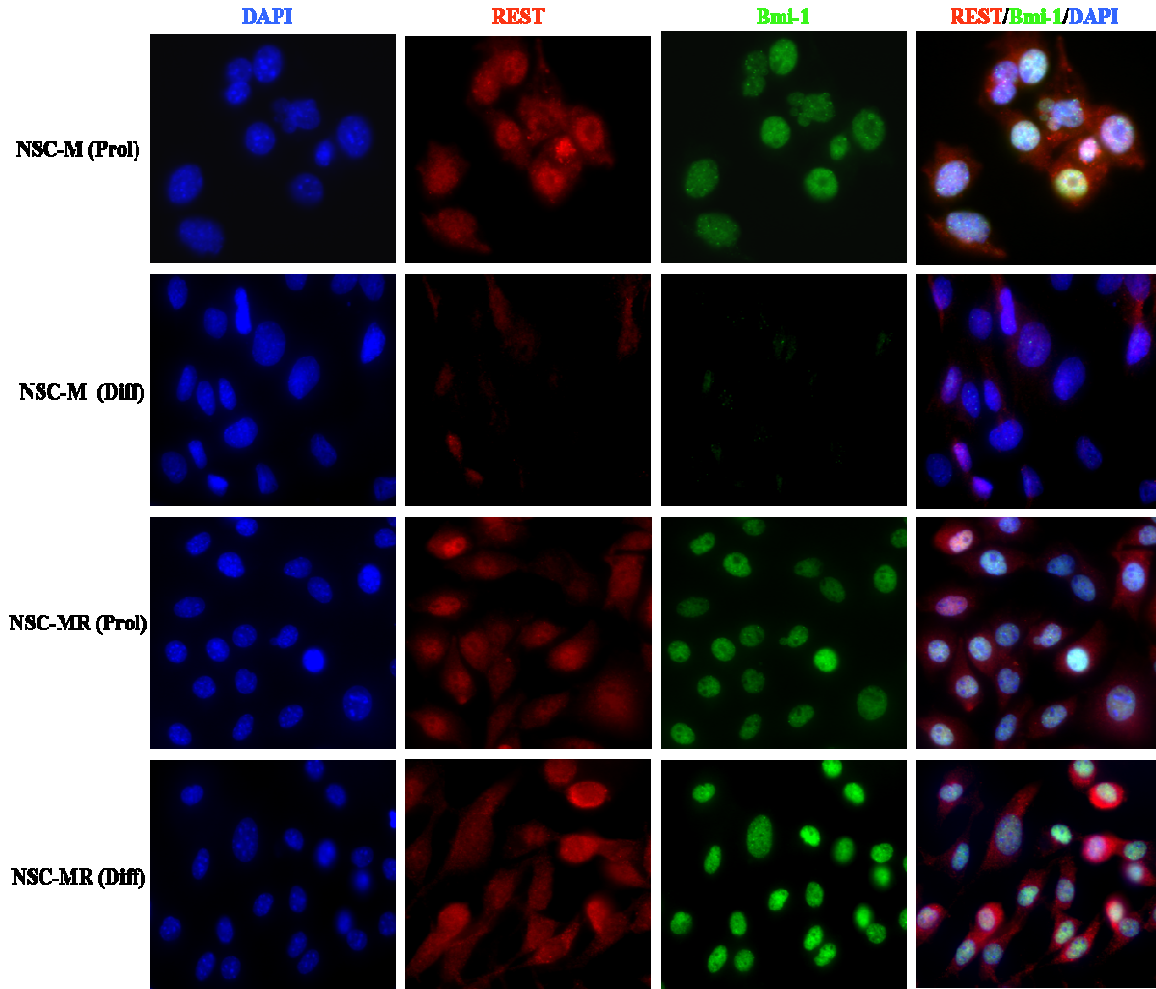


Figure 13: Constitutive REST expression maintains Bmi-1 levels. NSC-M and NSC-MR cells were cultured under proliferation or differentiation conditions. Cells were processed by IFA to detect changes in protein levels and sub-cellular localization of REST and Bmi-1 in NSC-M and NSC-MR cells. Cells were stained using commercially available anti-REST and anti-Bmi-1 antibodies. Nuclei were stained with DAPI. These images were captured using a Nikon microscope and analyzed with Metamorph software. Experiments were done in duplicate.

REST and Bmi-1 are expressed in the progenitor cells of the mouse cerebellum.

To determine if REST and Bmi-1 were concomitantly expressed in the proliferating cells of the EGL *in vivo*, REST and Bmi-1 protein levels were analyzed in the developing cerebellum of P7 and P12 mice. C57BL6 mice were mated and the brains of the resulting pups were harvested using IACUC approved protocols various days after birth and analyzed by IFA assays using commercially available anti-REST (red) or anti-Bmi-1 (green) antibodies. In P7 mice, REST and Bmi-1 are detected in the proliferating cells of the EGL but their levels are substantially reduced upon migration and they are barely detected in the IGL (Fig. 14). In P12 mice, the EGL is diminished to a few layers of progenitor cells and most cells in the cerebellum are now migrating post-mitotic and terminally differentiated neurons. REST and Bmi-1 are barely detected in any of the layers in P12 mice (Fig. 14).

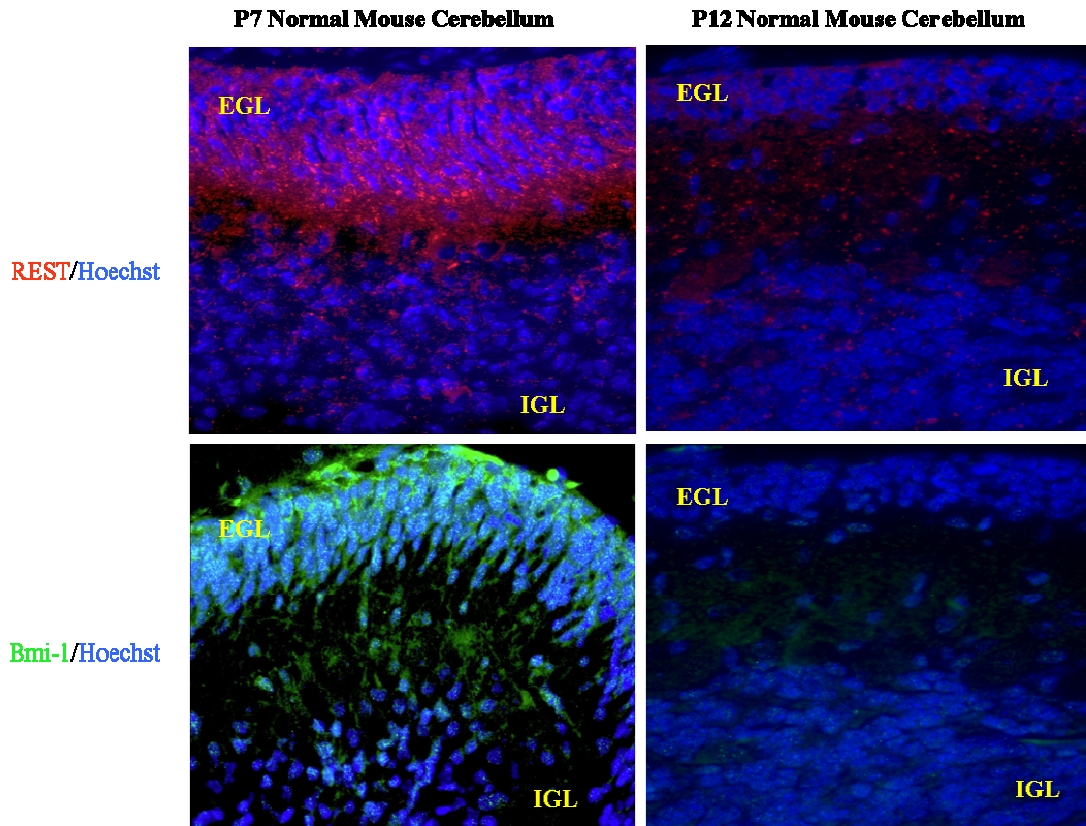


Figure 14: REST and Bmi-1 are expressed in the proliferating cells of the EGL. REST and Bmi-1 are concomitantly expressed in the EGL of the developing post-natal cerebellum of mice. C57BL6 mice were mated and the brains of the resulting pups were harvested using IACUC approved protocols various days after birth. REST and Bmi-1 protein in normal P7 and P12 mouse cerebellum was measured by immunofluorescence assay using commercially available anti-REST (red) and anti-Bmi-1 (green) antibodies. Nuclei were stained with Hoechst dye and representative images were captured using an Oil Immersion 40X lens on a Nikon fluorescence microscope. Images were analyzed with Metamorph software. Experiments were performed in duplicate. EGL- External Granule Layer, IGL-Internal Granule Layer.

REST binds to *miR-203* regulatory regions in NSC-M and NSC-MR cells.

To determine if *miR-203* was a direct target of REST, we performed chromatin immunoprecipitation (ChIP) assays on proliferating NSC-MRs and NSC-M cells, and differentiating NSC-Ms. Cross-linked nuclear extracts were immunoprecipitated using rabbit anti-REST antibodies or control rabbit immunoglobulin (IgG). Samples were analyzed by qPCR using primers specific to mouse *miR-203* and the sample values (REST) minus the control values (IgG) were used to determine relative levels of REST binding. We observed that REST bound to the regulatory region of *miR-203* in NSC-M and NSC-MR cells. Additionally, REST showed a significantly higher binding to the *miR-203* regulatory region relative to IgG controls in proliferating NSC-MR cells compared to NSC-M cells plated under the same conditions (Fig. 15). Furthermore, REST binding to *miR-203* was significantly higher in proliferating NSC-M cells compared to differentiated cells (Fig. 15). Together, these data suggests that *miR-203* may be a direct REST target.

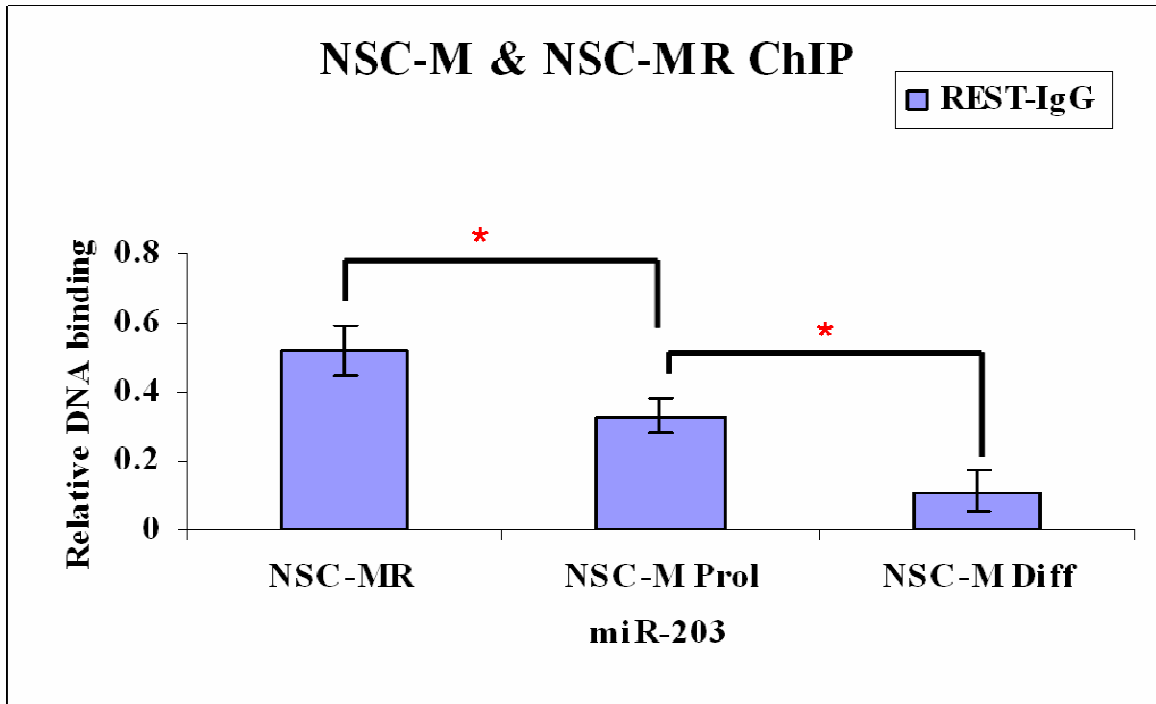


Figure 15: REST binds to the *miR-203* regulatory region in NSC-M and NSC-MR cells. Chromatin Immunoprecipitation (ChIP) assays were performed as follows: Cross-linked nuclear extracts prepared from proliferating NSC-MRs and NSC-M cells, and differentiating NSC-Ms were immunoprecipitated using rabbit anti-REST antibodies or control rabbit immunoglobulin (IgG). Samples were analyzed by qPCR using primers specific to mouse *miR-203* and the sample values (REST) minus the control values (IgG) were used to determine relative levels of REST binding. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

Summary

In this section, we investigated the mechanism by which REST regulates p16^{Ink4a} expression. We observed *miR-203* expression was lower in NSC-MR cells relative to NSC-M cells as detected by Taqman MicroRNA assay followed by qRT-PCR analyses. Additionally, ChIP analyses revealed REST binding to the *RE1* element in the downstream regulatory region of *miR-203*. Onset of neuronal differentiation in NSC-M cells was accompanied by a decrease in REST and Bmi-1 expression and an increase in *miR-203* expression. Maintenance of REST expression in NSC-MR cells blocked the differentiated associated decline in Bmi-1 levels and increased in *miR-203*, respectively. *In vivo*, IFA analyses showed REST and Bmi-1 protein at high levels in the EGL of the developing cerebella with their levels being substantially reduced upon migration to the ML and IGL.

Our findings suggested that REST may contribute to increased cell proliferation by repressing the expression of *miR-203*, a negative regulator of Bmi-1. Maintenance of Bmi-1 expression prevented p16^{Ink4a} transcription.

Chapter 4

Discussion

REST expression promotes cell proliferation in neural progenitor cells

REST is a negative regulator of neuronal phenotype and key regulator of brain development in vertebrates (13, 14, 19). It is a transcriptional repressor of neuronal differentiation genes. REST is expressed in ESCs, NSCs, and non-neural cells, but is downregulated in most neurons, consistent with its role as a negative regulator of neuronal gene expression (13, 14, 19, 21). In ESCs and NSCs, REST prevents the premature expression of terminal neuronal differentiation genes by binding to the *RE1* element in the regulatory regions of its target genes (13, 14). Thus, most recent studies have examined REST in the context of its function in the regulation of neuronal differentiation genes. Our recent studies are the first to attribute a novel role for REST in the control of cell proliferation and cell cycle exit (73). We observed that ectopic human REST transgene expression in NSC-Ms facilitated sustained proliferation, even under differentiation conditions (3). Conversely, *REST* knockdown in NSC-M cells caused a decline in cell numbers concomitant with a decrease in cell proliferation (73). Together, our studies suggest that REST may function as a molecular switch that coordinates cessation of cell proliferation with onset of neuronal differentiation (73). The underlying molecular mechanism(s) of REST regulating proliferation is the major goal of the current thesis project.

REST negatively regulates cell cycle exit

Consistent with a potential role for REST in the regulation of cell proliferation, it is expressed only in the G1 phase of the cell cycle (24). Cell cycle exit is one of the fundamental steps that must occur for cells to cease proliferating and begin expressing terminal differentiation markers required for a neuronal phenotype. Transition through

the cell cycle is governed by the activity of the cyclin/CDK complexes (40). These complexes promote phosphorylation of RB protein, inactivating it, and allowing progression through the cell cycle (40). The activity of CDK/cyclin complexes in turn is regulated by the CDKIs (40). Given the importance of CDKIs in cerebellar development, we examined if the expression of one or more of these CDKIs were subject to negative regulation by REST. In the current study, we have identified the CDKI, p16^{Ink4a}, as a downstream target of the REST pathway and suggest that REST-dependent control of cell proliferation may be achieved by repression of p16^{Ink4a} expression.

p16^{Ink4a} is a negative regulator of cell cycle progression

Work from several laboratories has linked p16^{Ink4a} to the regulation of cell proliferation in various progenitors and its dysregulated expression to oncogenesis (40, 55, 56, 68). p16^{Ink4a} expression is relatively low in proliferating neural progenitor cells with ubiquitous expression occurring only later in life when cells are terminally differentiated (52-54, 58). Its activation leads to growth arrest, senescence and apoptosis (52-54). In neural progenitors, its premature expression causes a significant decline in proliferation and reduces self-renewal of NSCs in culture (41-43). Interestingly, p16^{Ink4a} is necessary for cell cycle exit, but its expression alone is insufficient to induce neuronal differentiation (58). Other INK4a proteins, such as p15^{Ink4b}, are required to induce neuronal differentiation (58). The p16^{Ink4a} gene, located on human chromosome 9p21, is frequently deleted in many cancers including melanoma, glioblastoma, leukemia, and medulloblastoma, suggesting that it functions as a tumor suppressor (40, 55, 56).

REST and p16^{Ink4a} are negatively correlated *in vivo* and *in vitro* during differentiation of neural progenitor cells

In the current study, we have established a reciprocal pattern of expression between REST and p16^{Ink4a} in the developing postnatal cerebellum of mice. REST expression was detected almost exclusively in the proliferating progenitors of the EGL of P7 cerebella, a developmental stage at which maximal proliferation of progenitors is observed. REST levels decline as cells exit the cell cycle and initiate their migration down the ML to the IGL, where they become terminally differentiated neurons. Our findings are consistent with that by Ravanpay and colleagues, who also showed REST expression in the proliferating cells of the EGL (57). In contrast, p16^{Ink4a} was not found in the proliferating cells of the EGL, but showed significant expression in the post-mitotic and terminally differentiated neurons of the IGL. These observations are similar to findings by the Roussel group where they show p16^{Ink4a} to be expressed post-natally in neurons (58). In older P12 mice, the cerebellar EGL is largely depleted of progenitors with a vast majority of these cells having terminally differentiated into neurons of the IGL. Consistent with diminished proliferation rates of CPCs in the murine P12 cerebella, REST expression is detected only at very low levels in these cells. These findings are again similar to that described by the Olson group (57). The significant increase in p16^{Ink4a} levels in most post-mitotic cells of the P12 murine cerebellum is in line with its function as a CDKI.

These observations were recapitulated *in vitro* during neuronal differentiation of NSC-M neural progenitors. REST has been shown to be expressed in neural progenitors and be downregulated in neurons (13, 14, 19) while p16^{Ink4a} has been shown to be

expressed only in differentiated neurons (58). Our observations showed that downregulation of REST *mRNA* and protein levels coincided with upregulation of p16^{Ink4a} *mRNA* and protein levels during neuronal differentiation of NSC-M cells, consistent with previous studies. Interestingly, as seen by IFA, NSC-MR cells show REST to be localized in the cytoplasm as well as in the nucleus. This potentially suggests a role for REST in regulating itself through a negative feedback loop when its levels exceed a certain threshold. This feedback loop may cause REST to be degraded and therefore be expressed in the cytoplasm and may be a subject worth investigating. Collectively, these findings indicate a reciprocal relationship between REST and p16^{Ink4a}.

Furthermore, constitutive expression of human *REST* transgene in NSC-MR cells blocked upregulation of p16^{Ink4a} *mRNA* and protein levels, even under neuronal differentiation conditions. These findings suggest a potential role of REST expression in contributing to uncontrolled proliferation of NSC-MR cells by regulating p16^{Ink4a} expression. We further validated our findings by protein and sub cellular localization through IFA in NSC-M and NSC-MR cells and together, all of these findings recapitulated the reciprocal relationship between REST and p16^{Ink4a}. The mechanism by which this relationship occurs is discussed below and is a sub aim of the current thesis project.

REST indirectly regulates p16^{Ink4a} expression

REST is a transcriptional repressor that regulates expression of many neuron-specific genes by binding a motif called the *REI* element present in the regulatory regions of its target genes. Since our observations suggest that these changes in p16^{Ink4a} expression may be in response to differences in REST levels or activity during neuronal

differentiation, we first asked if REST had the potential to regulate p16^{Ink4a} transcription (13, 20, 21). We conducted a computational analysis of the p16^{Ink4a} locus for a consensus *RE1* binding site, however, this search did not reveal the REST binding motif, suggesting that the effect of REST on p16^{Ink4a} may be indirect.

Bmi-1 transcriptionally regulates p16^{Ink4a} expression

Previous studies have linked Bmi-1, a polycomb group gene and epigenetic regulator of gene expression (60, 61) to the transcriptional regulation of p16^{Ink4a} in CPCs. Bmi-1 mediated control of the Ink4a locus that encodes p16^{Ink4a} is important for the maintenance of proliferation and self-renewal potential in these progenitor cells as demonstrated by the following studies (59, 60, 62). In *Bmi-1* null (*Bmi-1*^{-/-}) mice, p16^{Ink4a} is precociously upregulated in NSCs and progenitor cells causing a reduction in self-renewal leading to a morphologically smaller cerebella, including significantly thinner granular and molecular cells layers (43). Loss of INK4a in *Bmi-1*^{-/-} mice prevents neurogenesis *in vivo* and partially rescued the defect in self-renewal, suggesting that repression of p16^{Ink4a} is necessary for stem cell self-renewal in the presence of Bmi-1 (42). These findings led us to hypothesize that REST-dependent transcriptional regulation of p16^{Ink4a} expression maybe mediated by Bmi-1.

REST maintains Bmi-1 expression by repressing *miR-203*

However, the absence of a *RE1* site in the upstream or downstream regulatory region of Bmi-1 gene makes it unlikely to be a direct target of REST. Interestingly, recent reports in the literature identified Bmi-1 as a target of microRNAs (miRs), specifically of *miR-15a* and *miR-16*, in ovarian cancer (48) and *miR-203* in pancreatic cells (49). MicroRNAs are important for the control of neurogenesis and neural cell fate

specification during brain development (64). Some of the most widely expressed miRs in the nervous system, including *miR-124* and *miR-9* (22, 31-33), are regulated by REST. This led us to suspect that REST-dependent maintenance of Bmi-1 expression may be through a miR known to negatively regulate Bmi-1. Our subsequent studies led us to identify *miR-203* as a direct REST target as measured by qRT-PCR analyses and CHIP assays. REST showed significant binding to the *miR-203* regulatory region relative to IgG controls in NSC-M and NSC-MR cells. Our observations are consistent with other studies that have demonstrated that *miR-203* interacting with REST in the mouse NSC line, NS5 (50). Additionally, *miR-203* has a stemness-inhibiting and pro-differentiation function in skin progenitor cells (65) and was previously shown to directly inhibit Bmi-1 expression in human pancreatic cancer cells (49). Finally, although we have focused on *miR-203* in our studies, *miR-183* expression also changed in a REST-dependent manner. However, the lack of a *RE1* motif suggests that its expression may be controlled by other intermediate REST target genes.

Previous work using mouse ESCs also showed that induction of neuronal differentiation was associated with a decrease in Bmi-1 levels and a reciprocal increase in *miR-203* expression (49). We observed a similar decline in *REST* and *Bmi-1*, and an increase in *miR-203* expression during neuronal differentiation of neural progenitors *in vitro*. This also corroborates other results from previous studies where *Bmi-1* expression is reduced (66) and *miR-203* expression increases as cells differentiate (49).

In contrast, constitutive-REST expressing NSC-MR cells blocked the upregulation of *miR-203* expression during neuronal differentiation and maintained *Bmi-1* expression. This data coincided with previous work that shows NSC-MR cells fail to

differentiate and have sustained proliferation (3). We further validated our findings by protein and sub cellular localization through IFA in NSC-M and NSC-MR cells. This provides the first connection between REST and Bmi-1 through *miR-203* and potentially with self-renewal of CPCs (50). Reporter assays will provide further *in vitro* confirmation of these relationships.

In vivo, we established a concomitant pattern of expression between REST and Bmi-1 in the developing postnatal cerebellum of mice. REST and Bmi-1 expression was detected almost solely in the proliferating progenitors of the EGL of P7 cerebella. REST and Bmi-1 levels decline as cells exit the cell cycle and initiate their migration down the ML to the IGL, where they become terminally differentiated neurons. In older P12 mice, REST and Bmi-1 expression are detected only at very low levels, consistent with diminished proliferation potential of CPCs in the murine P12 cerebella.

Our *in vivo* and *in vitro* studies described provide the basis for further investigations in the relationship between REST, *miR-203*, Bmi-1, and p16^{Ink4a} in the regulation of cell proliferation and self-renewal of neural progenitors in mice with conditional *REST* knockout in the cerebellum. *In vitro*, elevated expression of either *REST* or *Bmi-1* causes sustained proliferation of immortalized neural progenitors (3, 46). Hyper-proliferation is a hallmark of cancers and consistent with this, knockdown of *Bmi-1* or *REST* in human MB cell lines suppresses their tumorigenic potential in mouse orthotopic models (35, 67). Elevated levels of *REST* (unpublished data) or lower levels of p16^{Ink4a} (68) expression in tumor tissue of patients with MB have been independently associated with poor prognosis. Maintenance of REST and Bmi-1 leads to inhibition of *miR-203* and p16^{Ink4a} and may contribute to uncontrolled proliferation of progenitor cells

and potentially tumorigenic cells. It would be interesting to assess the involvement of *miR-203* and p16^{Ink4a} in the process of tumorigenesis in mouse models of the disease (3). Attenuation of REST signaling may have therapeutic implications and could be potentially achieved by upregulating *miR-203* in these tumors. Additional studies are needed to provide a better understanding of how deregulation of this pathway may contribute to tumor formation of MB.

Chapter 5

Conclusions and Future Direction

Conclusions

We have demonstrated that:

- 1. REST and p16^{Ink4a} are reciprocally expressed in the developing post-natal cerebellum of mice.**

We showed that in P7 mice, where maximal proliferation of CPCs is observed, REST is expressed in the proliferating cells of the expanding EGL but not in the differentiated neurons of the cerebellum. Conversely, p16^{Ink4a} was not found in the proliferating cells of the EGL and was only expressed in the post-mitotic and terminally differentiated neurons of the IGL. In P12 mice where cells are predominantly post-mitotic, REST levels are substantially reduced. In contrast, p16^{Ink4a} was detected in these post-mitotic and terminally differentiated cells of P12 mice.

- 2. REST and p16^{Ink4a} are reciprocally correlated *in vitro* during neuronal differentiation of progenitor cells**

We cultured NSC-M cells under proliferation or differentiation conditions and we observed downregulation of REST *mRNA* and protein levels coincident with upregulation of p16^{Ink4a} *mRNA* and protein levels upon onset of neuronal differentiation.

- 3. Constitutive REST expression in progenitors (NSC-MR cells) blocks upregulation of p16^{Ink4a}.**

We observed that maintenance of REST expression in NSC-MR cells enforced suppression of p16^{Ink4a} *mRNA* and protein expression in NSC-MR cells both under proliferation and differentiation conditions.

4. REST-dependent regulation of $p16^{Ink4a}$ appears to be indirect since $p16^{Ink4a}$ lacks a REST binding site.

5. The polycomb protein Bmi-1, a known repressor of $p16^{Ink4a}$ transcription is positively correlated with REST in proliferating progenitors and differentiated neurons in the murine cerebellum.

In P7 mice, REST and Bmi-1 are both expressed in the proliferating progenitor cells of the expanding EGL but not in the differentiated neurons of the IGL. In P12 mice, where most progenitors are post-mitotic, REST and Bmi-1 levels are substantially reduced or absent.

6. *In vitro*, the expression of Bmi-1 is maintained in proliferating REST-expressing CPCs and NSC-M cells but not in differentiated neurons where REST was absent as well.

We cultured NSC-M cells under proliferation or differentiation conditions and observed downregulation of REST *mRNA* and protein levels during neuronal differentiation, which coincided with a decline in Bmi-1 *mRNA* and protein level. Conversely, NSC-MR cells that express REST in a constitutive manner maintained Bmi-1 expression under proliferation and differentiation conditions.

7. *MiR-203*, a negative regulator of Bmi-1 expression shows a pattern of expression that is reciprocal to the expression of REST and Bmi-1 in proliferating and differentiating progenitors.

We cultured NSC-M cells under proliferation or differentiation conditions and observed downregulation of REST *mRNA* and protein levels during neuronal differentiation, which coincided with an increase in *miR-203* expression.

8. Constitutive REST expression blocked upregulation of *miR-203* in NSC-MR cells. We observed that constitutive REST expression in NSC-MR cells prevented upregulation of *miR-203* expression.

9. REST binds to the downstream regulatory region of *miR-203* in NSC-M and NSC-MR cells.

We showed that REST significantly binds to the *miR-203* regulatory region relative to control immunoglobulin in NSC-M and NSC-MR cells.

Future Directions

1. To investigate the correlation between REST, *miR-203*, Bmi-1 and p16^{Ink4a} in primary progenitors

In further validate our findings in NSC-M progenitors, our next step would be to see how REST and p16^{Ink4a} are correlated in primary progenitors grown under either proliferation or differentiation conditions. *miR-203* and Bmi-1 expression will also be assessed in these cells. We believe that REST and Bmi-1 *mRNA* and protein levels would decline under neuronal differentiation while *miR-203* and p16^{Ink4a} would be upregulated, confirming the reciprocal correlation seen in NSC-M cells.

2. To investigate *miR-203*, Bmi-1 and p16^{Ink4a} expression n mice with conditional knockdown of REST expression in CPCs.

To further investigate the effect of REST knockdown on p16^{Ink4a} expression and cell proliferation, mice lacking REST expression in the cerebellar EGL are being generated. The expression of Bmi-1 and p16^{Ink4a} will be examined by immunofluorescence assay using cerebella harvested from animals at various times after birth. The expression of these molecules as well as *miR-203* will be studied by qRT-PCR/Western blotting *in vitro* in dissociated primary progenitors. We anticipate that by inhibiting REST, Bmi-1 would also be downregulated while p16^{Ink4a} would be upregulated.

3. To examine the effect of *miR-203* overexpression on Bmi-1 and p16^{Ink4a} expression.

In our current study, we showed that REST overexpression causes a downregulation of *miR-203* levels. Therefore we propose that countering REST expression by overexpressing *miR-203* might abrogate proliferation of these cells through downregulation of Bmi-1 expression and upregulation of p16^{Ink4a}. NSC-M cells, NSC-MR cells, and primary progenitors will be transfected with *miR-203* or control non-specific oligonucleotides. First changes in cell numbers will be plotted. Next, cell extracts will be subjected to Western blotting and immunofluorescence assay. We expect that constitutive *miR-203* expression will cause a decline in proliferation and cell numbers through repression of Bmi-1 expression and consequent upregulation of p16^{Ink4a} levels.

4. To interrogate if *miR-203* is a direct target of REST using reporter assays.

We showed in the current study that REST binds the *RE1* element in the *miR-203* regulatory region, suggesting that it may be a direct REST target. We will use clone the *RE1* element from the *miR-203* locus into reporter constructs and luciferase assays will be performed to confirm REST-dependent changes in reporter gene expression in NSC-M and NSC-MR cells. The *RE1* site will also be mutated to abolish REST binding and changes in reporter gene expression will be evaluated in NSC-M and NSC-MR cells. We expect *miR-203* luciferase activity to be significantly lower in NSC-MR cells compared to NSC-M cells. Further, we expect *miR-203* luciferase activity to be upregulated when the *RE1* site is mutated.

5. To study the relationship between REST and p16^{Ink4a} in human medulloblastomas known to express REST at elevated levels.

A major goal of our laboratory is to investigate the oncogenic function of REST in medulloblastoma, which are undifferentiated, hyperproliferative cancer arising from the cerebellar granular progenitors. Our previous and ongoing studies have demonstrated elevated REST expression in human medulloblastoma samples and shown this to be associated with poor prognostic significance for patients with the disease (38, 35, Taylor et al., in preparation). We also showed REST to contribute to blockade of neuronal differentiation and to be important for tumor progression and maintenance using mouse orthotopic models (38). Our recent studies have implicated elevated REST expression in neural progenitors to the deregulation of cell proliferation (73). Based on the findings of this thesis project, we propose that elevated REST expression in medulloblastoma may repress *miR-203* expression and lead to failure in p16^{Ink4a}. We will use genetic and biochemical approaches in vitro and in vivo to test this hypothesis.

6. To evaluate the therapeutic potential of constitutive *miR-203* expression in REST-expressing medulloblastoma in mouse orthotopic models.

If *miR-203* expression is important for upregulation of p16^{Ink4a} transcription and inhibiting cell cycle progression, then its expression is also likely to abrogate the tumorigenic potential of REST-expressing human medulloblastoma cells, a postulate that will be tested in mouse orthotopic models.

Chapter 6
Materials and Methods

6.1 Cell Culture

The mouse multipotent C17.2 (NSC-M) cell line (3, 69) was derived from neonatal mouse cerebellum and was cultured at 5% carbon dioxide, at 37°C in medium containing Dulbecco's modified Eagle's medium, (DMEM), 10% fetal bovine serum (FBS), 5% heat inactivated horse serum (HS), 2 mM l-glutamine, and 1% antibiotic/antimycotic solution (PSF) (all from Invitrogen, Carlsbad, CA). For proliferation conditions, cells were plated at a low confluency (30%), grown in proliferation medium (DMEM, 10%FBS, 5% HS, 2mM l-glutamine, and 1% antiobiotic/antimycotic solution) and collected after a day. NSC-M cells are known to differentiate at high confluency, so for differentiation conditions, cells were plated at 50% confluency, allowed to grow in proliferation medium to 100% confluency and collected after 5 days. NSC-M cells engineered to constitutively express REST (NSC-MR) under a doxycycline regulable promoter (previously described in Su and Gopalakrishnan, 2006 (3)) were cultured in both proliferation and differentiation conditions as described above.

6.2 Total RNA Isolation

Total RNA was extracted from NSC-M and NSC-MR cells using Trizol reagent (Invitrogen, Carlsbad, CA) and Chloroform (Sigma-Aldrich, St. Louis, MO) according to the Trizol manufacturer's guidelines (Invitrogen, Carlsbad, CA). Briefly, media was removed and 1ml of Trizol reagent was used to disrupt the cell pellet. 0.2ml of chloroform was added to lyse the cells followed by centrifugation (Hettich Rotina 35R, Hettich Zentrifugen, Hettich, Germany) at 12,000g for 15 minutes (min) at 4°C. The aqueous upper phase was collected in a minicentrifuge tube, precipitated with 0.5ml of

Isopropanal, and centrifuged at 12,000g for 10 min at 4°C. The pellet was then washed with 1ml of 75% ethanol, centrifuged at 7,500g for 5 min at 4°C, dried and resuspended in RNase-free water. RNA concentration was determined by spectrophotometric analysis (Nanodrop 1000 spectrophotometer, Thermo Scientific, Rockford, IL) and stored at -80°C. All experiments were performed in triplicate.

6.3 Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from NSC-M cells and NSC-MR cells grown under proliferation or differentiation conditions as described in section 6.1. Complementary DNA (cDNA) was synthesized from 1µg of total RNA from NSC-M and NSC-MR using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). Briefly, 4µl of 4x iScript Reaction Mix, 1µl of iScript Reverse Transcriptase and 1µg of Total RNA were diluted in water according to the manufacturer's guidelines. Quantitative PCR (qPCR) reactions were done using iQ Syber Green Supermix (Bio-Rad Laboratories, Hercules, CA) and primers specific to mouse *REST*, *p16^{Ink4a}*, *Bmi-1*, and *Synapsin* (Integrated DNA Technologies, Coralville, IA). Gene expression was normalized to mouse *18S* RNA levels. Relative *mRNA* measurements were done using the Comparative $\Delta\Delta$ ct method as previously described (70). All experiments were performed in triplicate.

Primer sequences are as follows:

Table 1: Forward and Reverse Primers for qRT-PCR

	Forward Primer 5'-3'	Reverse Primer 5'-3'
Mouse <i>REST</i>	GTGCGAACTCACACAGGAGAACG	GCTTCTCACCTGAATGAGTCCGCATA
Mouse <i>p16^{Ink4a}</i>	GAGCAGCATGGAGTCCGCTGC	GTTGCCCATCATCATCACCTG
Mouse <i>Bmi-1</i>	CTGCCAATGGCTCCATGAA	TTCCGATCCAATCTGCTCTG
Mouse <i>Synapsin</i>	CTCATTCCCTCAGTATCCCTT	GAAATCACCCCTTTAGATGTAC
Mouse <i>18S</i>	GAACTCACGGAGGATGAGGTG	GTTGGCCAGAACCTGGCTGTA

6.4 Western Blotting

Pellets were collected from NSC-M cells and NSC-MR cells cultivated under proliferating/differentiating conditions as described in section 6.1. Pellets were resuspended in 100µl western blot (WB) lysis buffer (50mM potassium chloride (KCL), 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 5mM Tris-Chloride (Tris-Cl) (pH 7.5), 10% glycerol, 2mM ethylenediaminetetraacetic acid (EDTA), 1mM DTT, 1% Triton X-100, 0.4% octylphenoxypolyethoxyethanol (Igepal), 1% protease inhibitors (Halt, Thermo Scientific, Rockford, IL) diluted in water) followed by sonication (Misonix Sonicator 3000, Misonix Incorporated, Farmingdale, NY). Samples were then spun at 15,000g for 10 min at 4°C to pellet debris and the supernatant was collected. Protein concentration was determined using a 1:5 dilution in water of Commassie blue reagent (Bio-Rad Protein Assay Dye-Reagent concentrate, Bio-Rad Laboratories, Hercules, CA). 995µl of 1:5 diluted Commassie blue reagent was mixed with 5µl of Protein and analyzed by spectrophotometric analysis at 600 nm (Genesys 10-S, Thermo Electron Corporation, Waltham, MA). Samples were electrophoresed by denaturing polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS). Electrophoresed proteins were transferred to a Hybond-P membrane (GE Healthcare, Little Chalfont, UK) in Transfer Buffer (3.03g Tris, 14.4g Glycine, 100ml Methanol, and 900ml of water). Membranes were blocked with 5% milk in Phosphatase Buffered Saline (PBS) with 0.1% Tween and incubated with primary antibodies as followed: REST (1:1000, Millipore, Waltham, MA), Bmi-1 (1:1000, Millipore, Waltham, MA), Tuj1 (1:1000, Covance, Denver, PA), Actin

(1:20,000, Cell Signaling, Danvers, MA), p16^{ink4a} (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA). Blots were incubated with peroxidase conjugated secondary antibodies against rabbit or mouse immunoglobulins (IgGs) and the immunocomplexes were visualized using Super Signal West Dura Enhanced Chemiluminescence (ECL) reagent (Thermo Scientific Pierce, Rockford, IL) and a Kodak film developer (Eastman Kodak). All experiments were performed in triplicate.

6.5 Chromatin Immunoprecipitation (ChIP) Assay

First, we prepared two times cross linking and homogenization mix (XHM) was prepared using 100mM Hepes (pH 8.0), 280 mM Sodium Chloride (NaCl), 2mM EDTA (pH 8.0), 0.8% Igepal CA-630, and 0.4% Triton X-100 diluted in water to final volume. Next we prepared cross-linking buffer (XLB) using XHM diluted 1:2 and protease inhibitors diluted in water to final volume. Cells were divided into aliquots of 1 million cells and pelleted by centrifugation at 5,000 rpm for 1 min at 4°C and cross-linked using XLB mixed with 1% formaldehyde. Samples were mixed by inverting 10 times and incubated with rocking for 10 min at room temperature (RT). The cross-linking was neutralized by adding 1/10 total volume of 1.4 M Glycine and rocking for 5 min at RT. The nuclei were pelleted by centrifugation at 14,000 rpm for 1 minute and then resuspended in 1 ml of homogenization buffer (XHM diluted 1:2 and protease inhibitors diluted in water to final volume). The nuclei were pelleted by centrifugation at 14,000 rpm for 1 minute and then washed with 1 ml of Nuclear Wash Buffer (1:10 dilution of 10X Nuclear Wash Buffer (200 mM Tris-hydrochloride (Tris-HCL) (pH 8.0), 10mM EDTA (pH 8.0), and 1.5M NaCl) and protease inhibitors diluted in water to final volume) twice and centrifuged at 14,000 rpm for 1 minute.

Cross-linked NSC-M or NSC-MR cells were resuspended in sonication buffer (50 mM Tris-HCL (pH 8.0), 10mM EDTA (pH 8.0), 1% SDS, and protease inhibitors) and sonicated. The samples were centrifuged twice for 1 minute each at 14,000 rpm to remove debris. For each set, 10% was saved as input DNA and the remaining 90% was diluted 10-fold with ChIP dilution buffer (16.7 mM Tris-HCL (pH 8.0), 167 mM NaCl, 1.2 mM EDTA (pH 8.0), 1.1% Triton X-100, and protease inhibitors diluted in water to the final volume) and pre-cleared by incubation with Protein G magnetic beads for 2 hrs at 4°C with shaking. The samples were then centrifuged at 3,000 rpm for 1 minute. The pre-cleared supernatant was incubated with 5µg anti-REST antibody or 5µg control IgG for 12 hrs at 4°C with shaking followed by addition of Protein G magnetic beads for 2 hrs at 4°C with shaking. Beads were collected by centrifugation at 1,000 rpm for 1 minute and then washed once with the following buffers: Wash buffer I (10mM Tris-Cl (pH 8.0), 150mM NaCl, 2mM EDTA (pH 8.0), 0.1% SDS, 1% Triton X-100 diluted in water to the final volume); Wash buffer II (20mM Tris-Cl (pH 8.0), 500mM NaCl, 2mM EDTA (pH 8.0), 0.1% SDS, 1% Triton X-100 diluted in water diluted in water to the final volume); Wash buffer III (10mM Tris-Cl (pH 8.0), 0.25 M Lithium Chloride (LiCl), 1mM EDTA (pH 8.0), 1% Igepal CA630, 1% deoxycholate diluted in water to the final volume); Wash buffer IV (Tris EDTA (TE) (pH 8.0). After washing, the beads were then eluted with elution buffer (2%SDS, 0.2M Sodium Bicarbonate (NaHCO₃) diluted in water to the final volume) and vortexed at RT for 10 min. The beads were pelleted with a magnetic rack and supernatants moved to a new tube and mixed with 1 volume of 2X dilution buffer (20mM Tris-HCL (pH 8.0), 2mM EDTA (pH 8.0) diluted in water. Elution buffer (Diluted 1:2) was added to inputs. Cross-linking was reversed by adding

5M NaCl to a final concentration of 0.3M to the elutants and inputs and incubated at 65°C for 6 hours. The DNA was purified with a QiaQuick PCR Purification kit (Qiagen, Valencia, CA). Bound DNA was quantified using SYBR-Green qPCR analyses as previously described (Das et al., 2010) and analyzed with IQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Calculations following normalization to input values were done as described previously (71). Briefly, the highest value was set to 1 and control IgG values were subtracted from the target (REST) value. All experiments were performed in triplicate. Primers used as follows:

Table 2: ChIP Forward and Reverse Primers.

	Forward Primer 5'-3'	Reverse Primers 5'-3'
Mouse <i>miR-203</i> (10kb)	GAAGGCAGCAGACACATGGCT	GTCTGTGGTCGTGTAGCCTTA

6.6 Immunofluorescence assay (IFA)

NSC-M and NSC-MR cells were seeded in Lab-Tek II Chamber Slides (Thermo Fisher Scientific, Rochester, NY) under proliferation or differentiation conditions as described in section 6.1. Cells were fixed with 2% buffered paraformaldehyde, blocked (5% dry milk, 0.2% Triton-X 100, 1% Goat serum in PBS) and incubated with rabbit anti-REST (1:100, Millipore, Waltham, MA), mouse anti-Bmi-1 (1:100, Millipore, Waltham, MA), or mouse anti-p16 (1:150, Santa Cruz Biotechnology, Santa Cruz, CA) in PBS for 90 min. After washing with PBS three times for three min, the cells were incubated with Cy3-labeled goat anti-rabbit IgG (Amersham, Pittsburgh, PA) or Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, Carlsbad, CA) for one hour. They were then covered with Slowfade Gold antifade (Invitrogen, Carlsbad, CA) containing 1 µg/ml DAPI and sealed. Images were visualized using a Nikon Fluorescence microscope

(Nikon Inc., Melville, NY) and analyzed using Metamorph software (Molecular Devices, Downingtown, PA).

6.7 Immunohistochemistry (IHC)

Mouse cerebellar sections sample slides were placed on a hot plate for 20 min and then deparaffinized and hydrated. Briefly, slides were immersed in xylene for 4 min followed by a second treatment for 3 min. Then, the slides were immersed in 100% ethanol twice for 2 min, 95% ethanol twice for 1 min, 80% ethanol for 1 min followed by PBS twice for 2 min. The slides were then placed in citrate buffer (0.1M Sodium Citrate) and steamed for 25 min for antigen retrieval. Tissues were allowed to cool for 30 min, and then blocked (5% dry milk, 1% goat serum in PBS) for 20 min and incubated with rabbit anti-REST (1:50), mouse anti-p16 (1:150), or mouse anti-Bmi-1 (1:100) in blocking solution at 4°C over night in a humid chamber. Slides were washed in PBS and incubated with Cy3-or A488 conjugated secondary antibodies (1:800 in PBS, Invitrogen, Carlsbad, CA) and then covered with Hoescht 33342 (1:10,000 in PBS). Images were visualized using a Nikon Fluorescence microscope and analyzed using Metamorph software.

6.8 Taqman® assay to measure MicroRNA (miR) expression

Total RNA was isolated from cells using TRIZOL reagent as described in section 6.2. Endogenous mature microRNA (miR) levels were quantified using TaqMan® MicroRNA assay (Applied Biosystems, Carlsbad, CA). First, total RNA was reverse transcribed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). Briefly, miR specific cDNA was prepared using 100mM deoxynucleotide triphosphate (dNTPs), 50 U/μL MultiScribe Reverse Transcriptase,

10X Reverse Transcription buffer, 20 U/ μ L RNase inhibitor, miR primers (U6, *miR-203*, *miR-183*, *miR-221*, *miR-16*, *miR-15a*, and *miR-194*, Applied Biosystems, Carlsbad, CA) 5 μ g of total RNA and nuclease-free water and amplified using a thermal cycler as follows: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and 4°C indefinitely. This was followed by qPCR using TaqMan® Universal PCR Master Mix, No AmpErase® Uracil N-glycosylase (UNG) (Applied Biosystems, Carlsbad, CA). Briefly, cDNA (diluted 1:10 in water) was mixed with 20X TaqMan MicroRNA Assay, TaqMan 2X Universal PCR Master Mix, No AmpErase UNG, and nuclease-free water. This was then run on a 384 qRT machine (Applied Biosystems, Carlsbad, CA) for 10 min at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Relative abundance of miR fold difference relative to U6 small nuclear RNA (RNU6B) expression was done using the comparative $\Delta\Delta$ ct method as described earlier (72). All experiments were performed in triplicate.

6.9 Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee at The University of Texas M.D. Anderson Cancer Center. Mice were purchased from Jackson Laboratories (Strain C57BL6) and mated. The brains of the resulting pups were harvested various days after birth and fixed in paraformaldehyde and paraffin embedded. Tissue sections were analyzed by IFA assays as described in section 6.7.

6.10 Statistical Analysis:

We used STATISTICA 6.0, Anova one-way software to analyze qRT-PCR and CHIP data. For qRT-PCR, the treated values (differentiation values) minus the untreated

values (proliferation values) were used in a one-way analysis of variance, with post hoc analysis using the Fisher least significant difference (LSD) to determine significant differences. $P < 0.05$ was considered statistically significant. For CHIP, the sample values (REST) minus the control values (IgG) were used in one-way analysis of variance, with post hoc analysis using the Fisher LSD to determine significant differences. $P < 0.05$ was considered statistically significant.

Chapter 7

Appendix

Supplemental Figures

REST expressing cells blocked p16^{Ink4a} upregulation.

To examine the relationship between REST and p16^{Ink4a} during neuronal differentiation of neural progenitors *in vitro*, NSC-M cells were cultured under proliferation or differentiation conditions. Total RNA was collected and *mRNA* levels were quantified by qRT-PCR analyses. Relative *REST* and *p16^{Ink4a}* *mRNA* levels were determined following normalization to *18S mRNA* levels, and then normalization to proliferation values. Downregulation of *REST* gene expression during neuronal differentiation significantly correlated with upregulation of *p16^{Ink4a}* gene expression (Fig. 16). The terminal differentiation marker Synapsin1 (Syn1) was used as a positive control since it is an established REST target gene.

Conversely, NSC-MR cells cultured under these conditions maintained *REST* expression and failed to upregulate *p16^{Ink4a}* *mRNA* levels, even when cultured under neuronal differentiation conditions (Fig. 16). Together these findings suggest that maintenance of REST expression and a failure to upregulate p16^{Ink4a} expression may contribute to uncontrolled proliferation of NSC-MR cells.

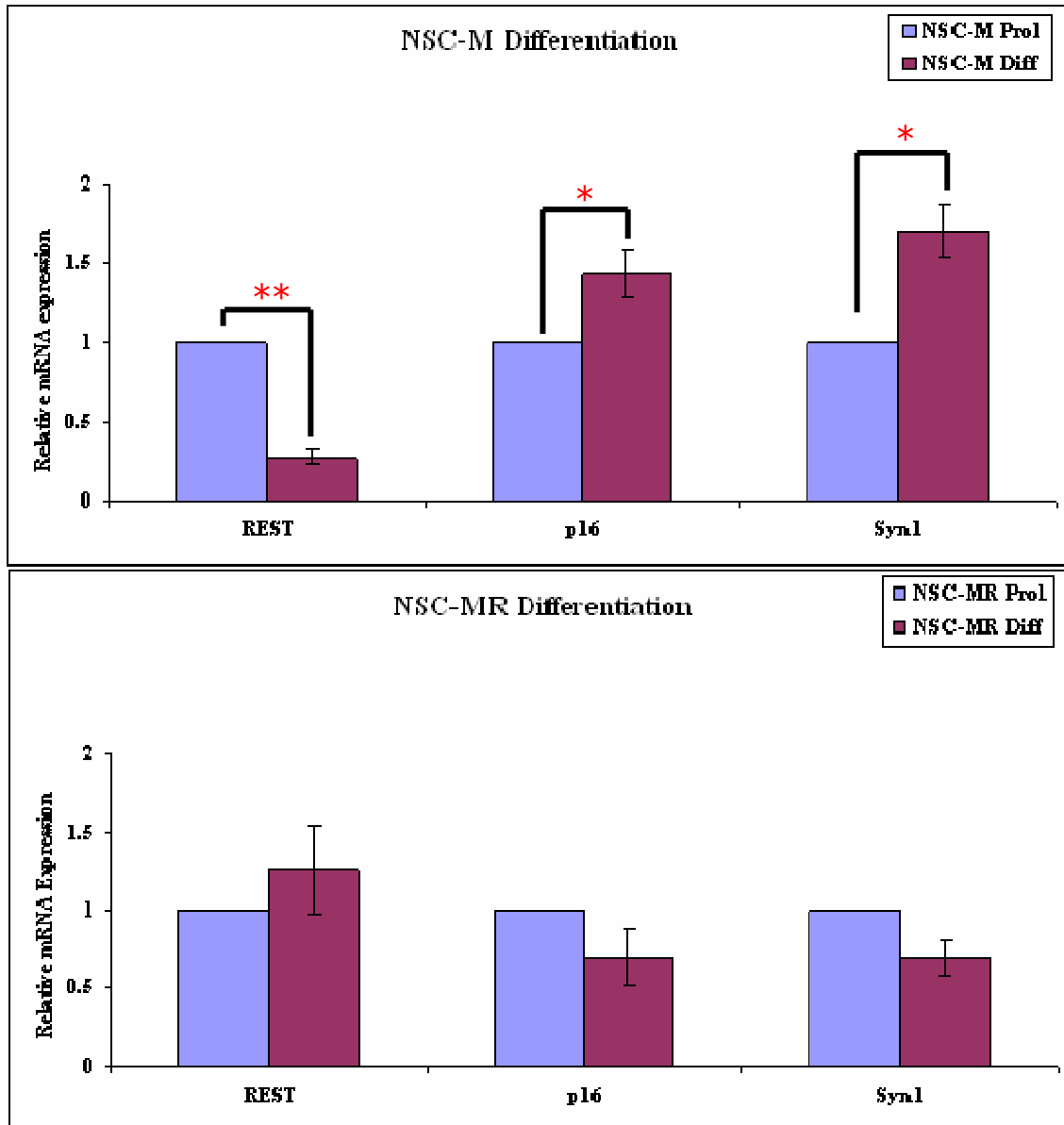


Figure 16: Maintenance of REST expression blocks $p16^{Ink4a}$ upregulation. NSC-M (top panel) and NSC-MR (bottom panel) cells were grown under proliferation or differentiation conditions. Total RNA was prepared, converted to cDNA, and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *REST*, $p16^{Ink4a}$ (p16), and *Synapsin1* (Syn1). Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (*-p<0.05, **-p<0.01).

REST-dependent Changes in *miR-203* expression.

NSC-M and NSC-MR cells were cultured under proliferation and differentiation conditions and examined by TaqMan MicroRNA assay followed by qRT-PCR analyses. Gene expression was determined following normalization to U6 small nuclear RNA (RNU6B) *mRNA* levels. As highlighted in Fig. 17, *miR-203* expression was significantly downregulated in REST overexpressing NSC-MR cells relative to NSC-M cells.

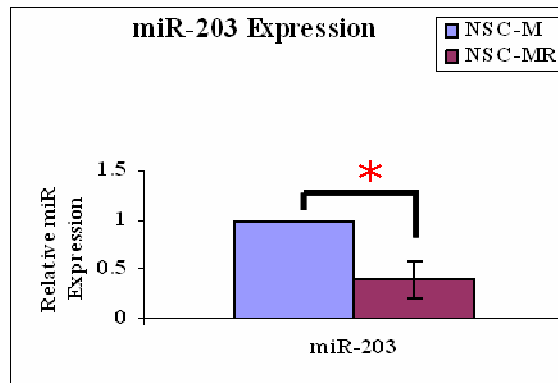


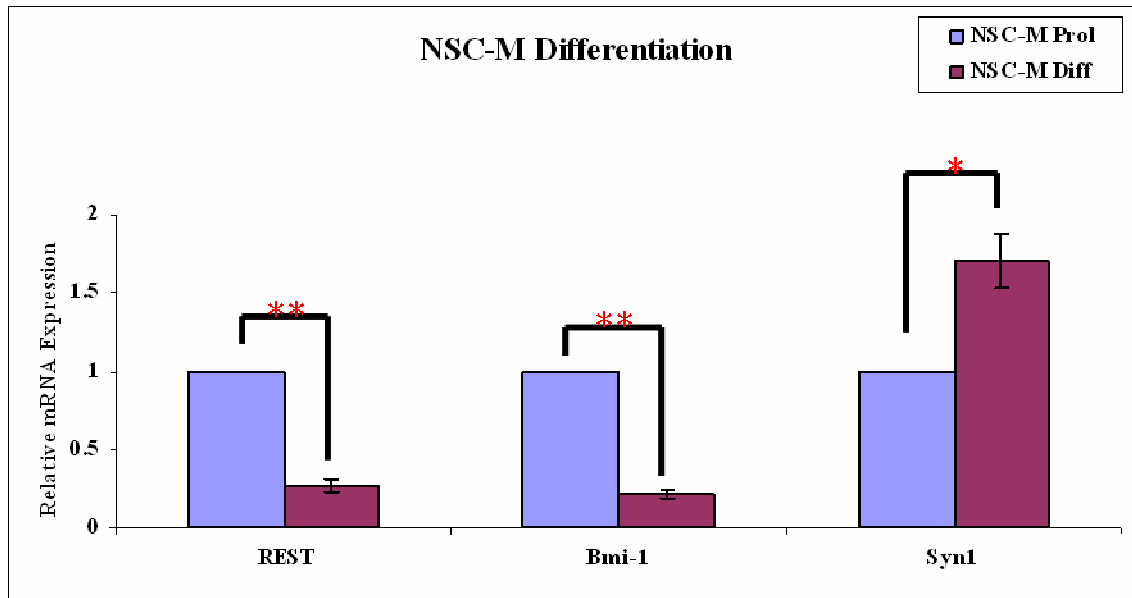
Figure 17: *MiR-203* and *miR-183* fluctuate in a REST dependent manner. NSC-M and NSC-MR cells were grown under proliferation conditions. Total RNA was prepared and miR expression was quantified by TaqMan MicroRNA assay followed by qRT-PCR analyses to measure changes in miR expression. *Mir-203* expression is significantly downregulated in NSC-MR cells relative to NSC-M cells. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

Constitutive REST expression blocked *miR-203* upregulation and maintained Bmi-1 expression in differentiated neural progenitors.

To better understand the relationship between REST, *miR-203*, and Bmi-1 expression we cultured NSC-M cells under either proliferation or differentiation conditions. Total RNA was collected, converted to cDNA and *18s*, *Bmi-1*, *Syn1*, and *REST* gene expression quantified by SYBR Green qRT-PCR. For *miR-203* measurements, total RNA was collected and *mRNA* levels were assessed by TaqMan MicroRNA assay followed by qRT-PCR analyses. *REST* gene expression (normalized to 18S expression, and then proliferation values) and *miR-203* (normalized to *RNU6B* expression, and then proliferation values) *mRNA* levels were found to be reciprocally correlated in differentiating NSC-M cells (Fig. 18). This shows downregulation of *REST* gene expression during neuronal differentiation caused a significant upregulation of *miR-203* gene expression (Fig. 18). The terminal differentiation marker Synapsin (*Syn1*), a known REST target gene was upregulated under these conditions and was used as positive control. Importantly, *Bmi-1 mRNA* levels were downregulated concomitant with a decline in *REST mRNA* levels during neuronal differentiation (Fig. 18).

In contrast, maintenance of REST expression in NSC-MR cells blocked the upregulation of *miR-203 mRNA* levels, even when cultured under differentiation conditions (Fig. 19). This correlated with maintenance of Bmi-1 *mRNA levels* in these cells (Fig. 19).

A.



B.

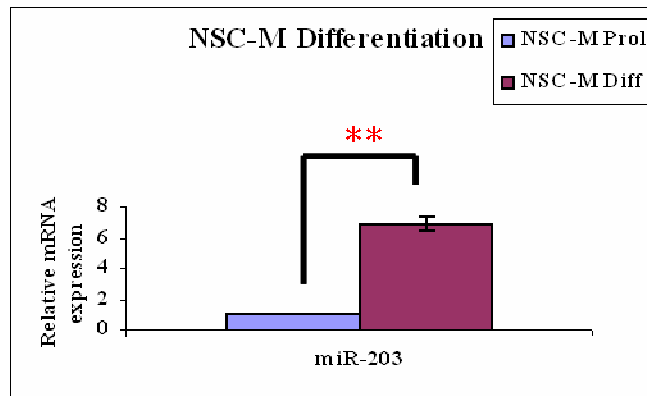


Figure 18: *REST* gene expression and *miR-203* mRNA levels were found to be reciprocally correlated in differentiating NSC-M cells. NSC-M cells were grown under proliferation or differentiation conditions. A.) Total RNA was prepared, converted to cDNA and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *REST*, *Bmi-1*, and *Synapsin1* (*Syn1*). B.) Total RNA was prepared, and mRNA levels assessed by Taqman MicroRNA assay followed by qRT-PCR analyses to measure changes in gene expression of *miR-203*. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

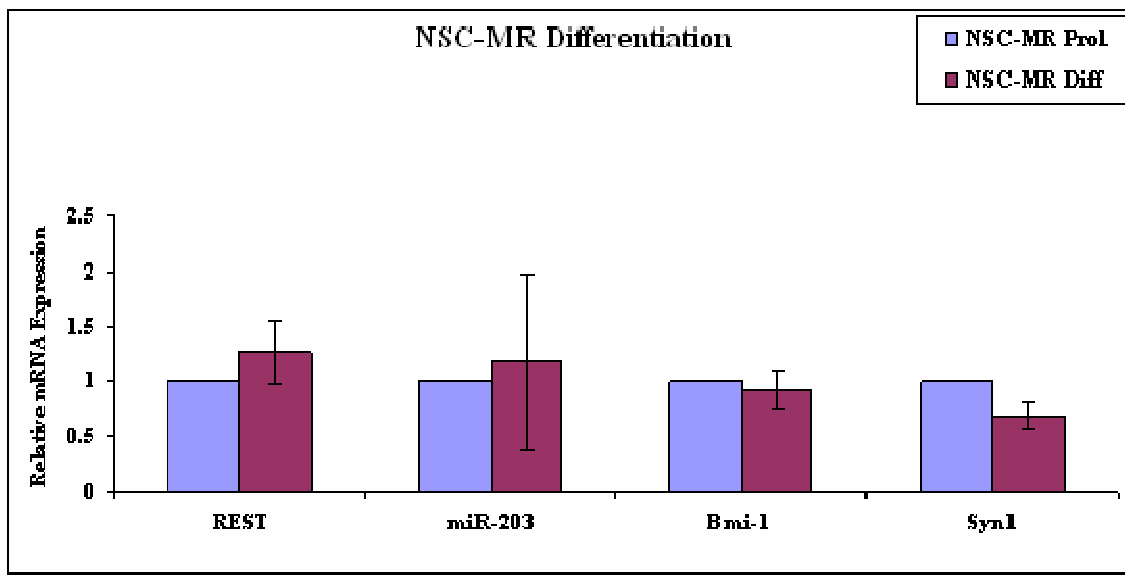


Figure 19: Maintenance of *REST* expression blocks *miR-203* upregulation and maintains *Bmi-1* expression. NSC-MR cells were grown under proliferation or differentiation conditions. Total RNA was prepared, converted to cDNA and analyzed by SYBR Green qRT-PCR to measure changes in gene expression of *REST*, *Bmi-1*, and *Synapsin1* (*Syn1*). For *miR-203*, total RNA was prepared, and *mRNA* levels assessed by Taqman MicroRNA assay followed by qRT-PCR analyses to measure changes in gene expression. Experiments were done in triplicate, and error bars are indicated. Statistical analysis was done using Statistica 6.0 (* $p < 0.05$, ** $p < 0.01$).

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VITA

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