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# P107 Negatively Regulates the Neural Precursor Pool by Repressing Hes1 Transcription

**by Crystal A. Wylie**

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

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## Table of Contents

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## Abstract

Stem cells are defined by their multipotentiality and their long-term ability to self-renew. P107, a member of the pocket protein family of cell cycle regulators has previously been shown in our laboratory to negatively regulate neural precursor cell number and self-renewal (Vanderluit et al., 2004). In this study, we investigated the mechanism by which p107 regulates the neural precursor pool by examining interactions between p107 and the Notch pathway, which has also been shown to regulate the neural stem cell population (Nakamura et al., 2000; Ohtsuka et al., 2001; Hitoshi et al., 2002b). We found an increase in both the transcript and protein levels of Hes1 in p107-/- brains using *in situ* hybridization and western blot analysis. Examination of the Hes1 promoter revealed three putative E2F binding sites, which were subsequently found to bind E2F3 and E2F4 using chromatin immunoprecipitation. P107 was found to significantly repress Hes1 promoter activity in the luciferase reporter assay, and finally, using the primary neurosphere assay we showed that removal of Hes1 from p107 -/- neurospheres restores the number of neurosphere forming cells to wildtype levels. Our results suggest that p107 represses Hes1 transcription through E2F, and demonstrate that an upregulation of Hes1 is responsible for the increased neural precursor pool in p107-/- mice.

## List of Figures

## List of Tables

## List of Abbreviations

BCIP - x-phosphate/5-bromo-4-chloro-3-indolyl-phosphate

BSA - bovine serum albumin

CDK - cyclin dependent kinase

ChIP - chromatin immunoprecipitation

DEPC - diethylpyrocarbonate

DIG - digoxigenin

D-MEM - Dulbecco's minimum essential medium

DMSO - dimethylsulfoxide

DNA - deoxyribonucleic acid

DTT - dithiothreitol

EDTA - ethylenediamine tetra-acetic acid

EGF - epidermal growth factor

FGF - fibroblast growth factor

HBSS - Hank's Buffered Saline Solution

HDAC - histone deacetylases

HEK 293 - human embryonic kidney 293

HERP - HES related proteins

HES - Hairy and Enhancer of Split

kb - kilobase

kDa - kilo Dalton

MOPS - 3-(N-morphine) propanesulfonic

mRNA - messenger ribonucleic acid

MUG - 4-methylumbelliferyl-D-galactoside

NBT - 4-nitro blue tetrazolium chloride

NICD - Notch Intercellular Domain

PAGE - polyacrylamide gel electrophoresis

PBS - phosphate buffered saline

PCR - polymerase chain reaction

PFA - paraformaldehyde

pRB - retinoblastoma (protein)

PVA - polyvinyl alcohol

RBP-Jk - Recombination Signal Binding Protein - Jk

RNA - ribonucleic acid

rpm - revolution per minute

RT – room temperature

SDS - sodium dodecyl sulphate

SEM - standard error of the mean

TAE - Tris/acetate buffer; 1X working solution containing 40mM Tris acetate and 2nM EDTA

Tris - tris(hydroxymethyl)aminomethane

TBS/T - Tris-buffered saline, Tween-20

TE - Tris - EDTA

# Introduction

## I. Neural Stem Cells and Progenitors

### *Overview*

Stem cells are defined by their ability to self-renew long-term, and to generate multiple differentiated cell types (multipotentiality) (Hall et al., 1989; Potten et al., 1990; Weissman et al., 2000). Tissue specific stem cells exist in the embryo in several organs and have also been shown to persist into adulthood in several tissues including the brain (Reynolds et al., 1992a; Tropepe et al., 2000; Toma et al., 2001; Okano et al., 2002). Neural stem cells are able to give rise to all of the major cell types in the brain including neurons, astrocytes and oligodendrocytes. Embryonic neural stem cells first appear in the mouse embryo around embryonic day (E) 8.5 as fibroblast growth factor 2 (FGF2) responsive, multipotent, self-renewing cells, derived from primitive neural stem cells (Tropepe et al., 1999).

There are several differences between neural stem cells and progenitor cells. Progenitor cells are often not multipotent, and in the few cases where they are, their multipotentiality is transient. Progenitors are also very limited in their ability to self-renew, unlike stem cells, which have long-term self-renewal capability (Seaberg et al., 2003). There are currently no markers that differentiate between neural stem cells and the more restricted progenitor cells, so the two different cell types must be identified and distinguished retrospectively by their functional properties. To assess multipotentiality isolated neural stem cells can be induced to differentiate, under differentiating conditions, into neurons, astrocytes, and oligodendrocytes. Self-renewal ability can be assessed using the in vitro neurosphere assay (Reynolds et al., 1992a; Campos, 2004).

### ***Embryonic Neural Stem Cells***

Undifferentiated, proliferating neural stem cells and progenitors reside in the ventricular zone of the telencephalon as a heterogeneous population. Radial glia emerge around E13-E14 and persist until birth in the rodent (Harfuss et al., 2001; Gotz et al., 2005). It has recently been shown that these cells can function as neural progenitors (Malatesta et al., 2000; Noctor et al., 2001; Noctor et al., 2002). They have radial morphology with long processes extending from the ventricular surface to the pial surface, while their cell bodies reside in the ventricular zone (reviewed in Gotz et al., 2005). It is along these processes that newly born neurons migrate to the appropriate layer in the cortex (Monuki et al., 2001). Radial glia express markers also expressed by mature astrocytes such as GLAST, a glutamate transporter, brain-lipid-binding protein (BLBP), and tenascin C (TN-C) (Malatesta et al., 2000; Malatesta et al., 2003; Gotz, 2003), which is why they were originally designated with glial character. Recently the validity of assigning glial character to radial glia has been questioned as researchers strive to determine the lineage of cells comprising the cortex (Parnavelas et al., 2001; Ever et al., 2005). It remains to be determined if these cells share functional characteristics with astrocytes in addition to their shared markers.

During development, neural stem cells acquire both positional and temporal information. For example Hitoshi et al. (2002a) found that neural stem cells isolated from different areas of the brain including the cortex, the ganglionic eminence and the midbrain/hindbrain expressed different regional specific markers that identified positional information along both the anterior-posterior axis as well as the dorsal-ventral axis. Zappone et al. (2000) found that stem cells isolated from the telencephalon expressed Sox2, however neural stem cells isolated from the spinal cord did not. In addition, studies have found that stem cells isolated from different parts of the brain produce progeny with region specific characteristics (Anderson et al., 1997; Kalyani et al., 1998; He et al., 2001). For example neural stem cells isolated from the basal forebrain and the dorsal forebrain generate different progeny even when cultured under identical conditions *in vitro* (He et al., 2001). These studies demonstrate that neural stem cells are not identical throughout the central nervous system, but instead contain positional information that is most likely important for the genesis of region specific neurons throughout the brain.

Not only do neural stem cells possess positional information, they also acquire temporal information. Qian et al. (2000) found that neural stem cells studied *in vitro* with time-lapse video microscopy and immunohistochemistry first produced neurons and later gave rise to glia. This mimics what happens *in vivo* as neurogenesis precedes gliogenesis during development (reviewed in Monuki et al., 2001). This study suggests that neural stem cells acquire temporal information, and that this information is important for timing of cell type production during cortical development.

In addition, stem cells change their responsiveness to growth factors over time. Neural stem cells responsive to FGF2 appear at E8.5 (Tropepe et al., 1999). At E14.5 there is a heterogeneous population of both epidermal growth factor (EGF) and FGF responsive neural stem cells derived from the earlier FGF only responsive cells (Reynolds et al., 1992b; Reynolds et al., 1996; Kilpatrick 1995; Tropepe et al., 1999). These studies show that neural stem cells change over time. These time dependent changes are most likely a key factor in the complex process that results in the genesis of different types of cells at different times during development.

### ***The Regulation of Neural Stem Cell Self-renewal***

A very important, defining characteristic of all stem cells is their ability to self-renew. The complete repertoire of intrinsic and extrinsic factors that regulate neural stem cell self-renewal in the embryonic brain is still being elucidated, however, we are beginning to understand some of the key genes and pathways involved. Some of the known factors involved are the Notch pathway (Nakamura et al., 2000; Ohtsuka et al., 2001; Hitoshi et al., 2002b; Yoon et al., 2004) and growth factors (Gensburger et al., 1987; Murphy et al., 1990; Drago et al., 1991; Kilpatrick et al., 1993). There have also been reports recently of Bmi-1 (Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky et al., 2005), and endothelial cells (Shen et al., 2004) playing a role in self-renewal.

The growth factors FGF2 and EGF are both important for neural stem cell proliferation and survival (Gensburger et al., 1987; Murphy et al., 1990; Drago et al., 1991; Kilpatrick et al., 1993). FGF2 responsive neural stem cells appear in the telencephalic germinal zone at E8.5 (Tropepe et al., 1999). FGF2 signalling is mediated mainly by FGF receptor1 (Johnson et al., 1993), although small amounts of FGF-receptor 2 have also been detected early in the brain (Weise et al., 1993). Later in cortical development (E13-E14) a population of EGF responsive neural stem cells arises (Reynolds et al., 1992b; Reynolds et al., 1996; Kilpatrick 1995; Tropepe et al., 1999). The EGF signal is mainly mediated by the EGF receptor (reviewed in Wong et al., 2004).

Bmi-1 has also recently been shown to regulate neural stem cell self-renewal (Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky et al., 2005). Cells isolated from the Bmi-1 (-/-) E14.5 mouse telencephalon gave rise to fewer multipotent primary and secondary neurospheres demonstrating that Bmi1 -/- mice have a smaller stem cell pool and that the self renewal ability of the stem cells is reduced (Molofsky et al., 2003). P19Arf and p16INK4a have been shown to act downstream of Bmi1 and removal of either p19Arf or p16INK4a from Bmi-1 -/- mice partially restores stem cell number and self-renewal ability (Molofsky et al., 2003; Bruggeman et al., 2005; Molofsky et al., 2005).

The Notch pathway is also very important for neural stem cell self-renewal (Nakamura et al., 2000; Ohtsuka et al., 2001; Hitoshi et al., 2002b; Yoon et al., 2004). Mice lacking members of the canonical Notch pathway such as Notch1, presenilin1, presenilin2, or RBP-Jk, have fewer stem cells and the existing stem cells show reduced self-renewal (Hitoshi et al., 2002b; Yoon et al., 2004). In addition, the downstream Notch effectors, Hes1 and Hes5, are also important for self-renewal (Nakamura et al., 2000; Ohtsuka et al., 2001). When Hes1 and Hes5 are removed there are fewer primary and secondary neurosphere forming cells (Ohsuka et al., 2001). These studies show that the Notch pathway is essential for neural stem cell self-renewal.

Recently endothelial cells, which line the basement membrane in capillaries, have been shown to affect neural stem cell self-renewal (Shen et al., 2004). Neural stem cells co-cultured with endothelial cells have increased self-renewal and show an upregulation of the Notch-Hes1 pathway. This study demonstrates that endothelial cells are an important component of the stem cell niche in both the embryonic and the adult brain (Shen et al., 2004).

The study of neural stem cells is important for understanding brain development and for the potential therapeutic use of these cells in the future. It is now accepted that the adult mammalian brain contains stem cells (Reynolds et al., 1992a). To harness the potential of these endogenous cells it is imperative that we understand the conditions and genetic pathways that are responsible for self-renewal and multipotentiality in the embryo. Understanding these mechanisms will allow for the replication of these conditions in the adult brain for therapeutic purposes in the treatment of neurodegenerative disease and brain injury.

## II. The Notch Pathway

### *Notch Pathway Overview*

The Notch pathway has been implicated in several developmental processes including inhibition of differentiation (Kumano et al., 2001; Mizutani et al., 2005), pattern formation (Conlon et al., 1995), cell specification (Kim et al., 1996), apoptosis (Deftos et al., 1998), and axon guidance (Crowner et al., 2003). Our understanding of the Notch pathway has grown immensely over the last decade and we now have an appreciation for the intricacy, complexity, and most importantly, the context specificity of the pathway. The mechanisms behind this context specificity are still unknown and this aspect will be a focus of future Notch pathway research.

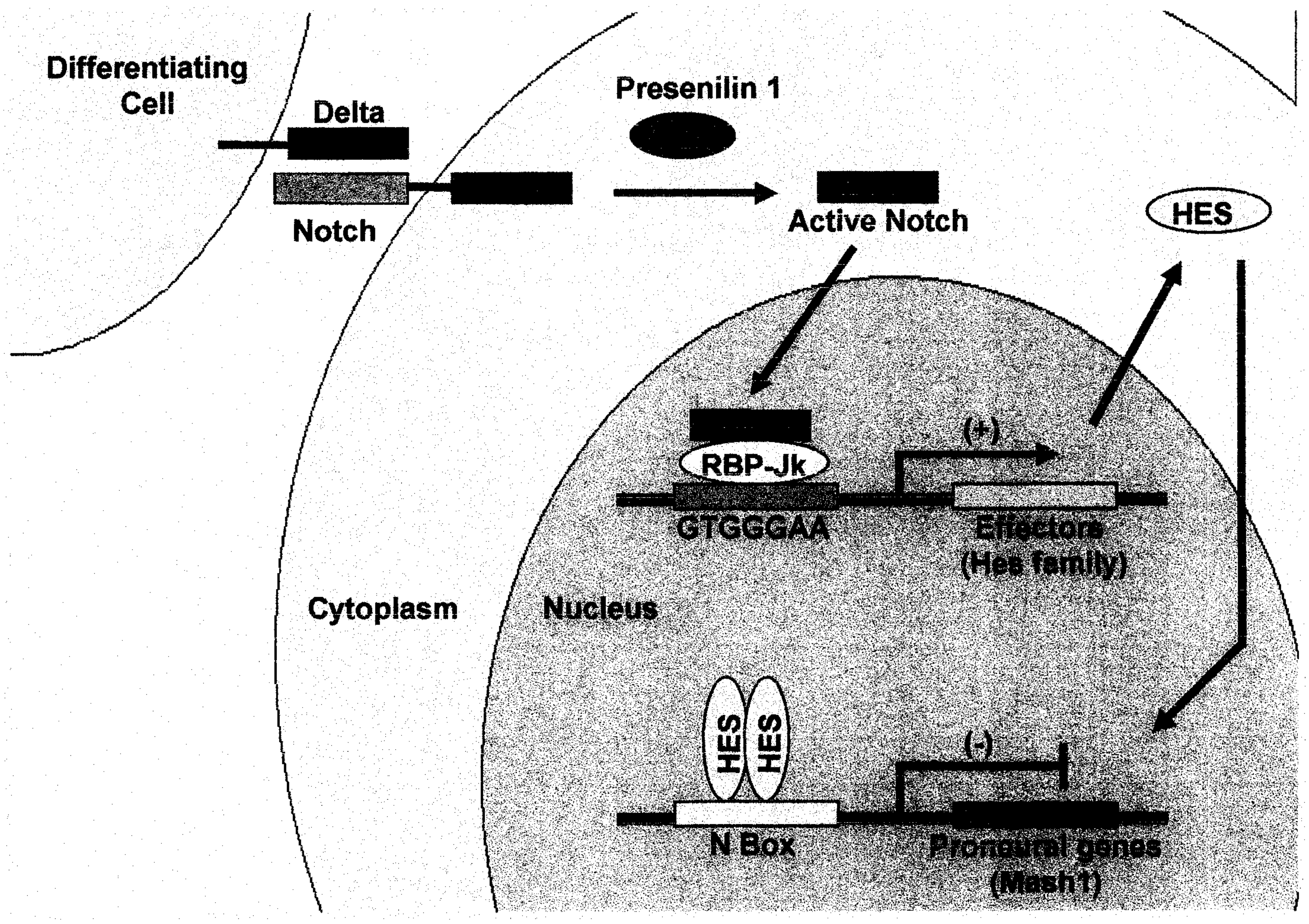
Notch was first discovered in *Drosophila melanogaster* where a mutation in the Notch gene produced a fly with a notched wing phenotype (Mohr, 1919). There are four known vertebrate homologs of Notch (Notch1-4), which encode cell surface transmembane receptors. Ligands for Notch include the Delta-like family and Jagged/Serrate family, which are themselves membrane bound (reviewed in Baron, 2003). The fact that both the receptors and ligands are membrane bound highlights one of the key characteristics of this pathway as involving cell-cell interaction.

The extracellular component of the Notch receptor contains a chain of 36 epidermal growth factor (EGF) repeats (Wharton et al., 1985). Specific EGF repeats interact with the Delta:Serrate:LAG-2 (DSL) domain present on the extracellular portion of Notch ligands (Rebay et al., 1991). Once the Notch receptor binds to a ligand, the intracellular portion of Notch is cleaved by the presenillin family (Struhl et al., 1999), which gives rise to the activated form of Notch, referred to as the Notch Intracellular Domain (NICD) (Figure 1). The NICD translocates to the nucleus where it interacts with the Recombination Signal Binding Protein - Jk (RBP-Jk) (also known as CSL or CBF1) to induce the transcription of downstream effector genes (Jarriault et al., 1995). RBP-Jk is bound to the regulatory sequences of Notch effector genes in the absence of NICD where it acts as a transcriptional repressor as part of a repressor complex that also includes histone deacetylase (Kao et al., 1998). Once the NICD enters the nucleus it replaces the repressor complex with a transcriptional activating complex that includes histone acetyltransferase p300 (Wallberg et al., 2002), mastermind (Wu et al, 2000), and RBP-Jk (Jarriault et al., 1995).

NICD and RBP-Jk initiate the transcription of several target genes, including the Hes (homolog of *Drosophila* Hairy and Enhancer of Split genes) (Jarriault et al., 1995; Ohtsuka et al., 1999: Bessho et al., 2001) and HERP (Hes Related Proteins) families (Iso et al., 2001a; Iso et al., 2002). It is important to point out that not all genes in these families appear to be Notch targets. In the central nervous system, Hes1 and Hes5 are the most widely studied Notch signalling effectors (Ishibashi et al., 1995; Ohtsuka et al., 1999; Nakamura et al., 2000; Hatakeyama et al., 2004).

The ligand (Delta-like or Jagged), receptor (Notch), and transcription factor (RBP-Jk) make up the core components of the Notch pathway. Homologs of the core Notch

Figure 1. **The Notch Signalling Pathway.** The Notch receptor binds to a ligand (Delta) on a neighboring cell. This results in the intercellular portion of Notch being cleaved to give the active form of Notch (NICD). NICD translocates into the nucleus where it forms a transcriptional activating complex with RBP-Jk (Recombinatin Signal Binding Protein-Jk) and initiates the transcription of Notch effector genes such as the members of the Hes family. The Hes family form dimers with other Hes family member or with HERP (Hes Related Protein) which bind N boxes, and inhibit the transcription of proneural genes such as Mash1.



Adapted from Iso et al., 2003. Journal of Cellular Physiology. 194:237-255 pathway have been found in all studied animals from nematodes to humans (Lai et al, 2004). This core pathway involved in numerous developmental processes throughout the animal kingdom. There are exceptions to this core structure (Henkel et al., 1994; Axelrod et al., 1996; Hu et al., 2003), however, understanding the core pathway is essential to understanding basic Notch signalling.

### *The basic Helix-Loop-Helix (bHLH) family of Transcription Factors*

The bHLH family of transcription factors are crucial for the development of several organs including the heart (Firulli, 2003), brain (Kageyama et al., 2005), pancreas (Schwitzgebel, 2001), and skeletal muscles (Cole et al., 2004). They have been extensively studied in the development of the mammalian cortex (reviewed in Ross et al., 2003), where they participate in proliferation (Ohtsuka et al., 1999; Nakamura et al., 2000; Mehler, 2002), cell specification (Fode et al., 2000; Yun et al., 2002; Zhou et al., 2002), and differentiation (Casarosa et al., 1999; Nakada et al., 2004). This family shares a basic Helix-Loop-Helix (bHLH) motif, which is important for its function. The basic region interacts with DNA and is involved in binding specificity and the HLH region interacts with other bHLH family members to form either homodimers or heterodimers, which then bind to DNA (Murre et al., 1994). The bHLH family is divided into three classes: A, B, and C (Fisher et al., 1998). Class A bHLHs are transcriptional activators that bind E boxes and include Mash1 (Nakada et al., 2004) and MyoD (Wyzykowski et al., 2002). The class B bHLHs have a characteristic leucine zipper and are also considered transcriptional activators. This class includes Myc (Oster et al., 2003) and Max (Jean-Francois et al., 2003). The Hes and HERP families belong to Class C, and are transcriptional repressors (Iso et al., 2003).

In terms of cortical development, there are several stages during which groups of bHLHs are important for controlling either proliferation or differentiation of the major cell types in the brain. The first stage is neural precursor proliferation during which Hes and Id are expressed in the ventricular zone (Akazawa et al., 1992; Sasai et al., 1992; Jen et al., 1997; Allen et al., 1999). This is followed by the neurogenesis stage between E12 and E17, which is initiated by the proneural bHLHs (Casarosa et al., 1999; Ma et al., 1996). The gliogenesis stage comes next followed by the formation of oligodendrocytes (reviewed in Sauvageot et al., 2002). The different temporal and spatial expression patterns of the bHLHs reflect what developmental process is taking place in the cortex.

Hes and Id genes are responsible for maintaining progenitors in an undifferentiated state (Ohtsuka et al, 2001; Mehler, 2002). The Hes family can repress proneural genes such as Mash1 by directly inhibiting their transcription (Chen et al, 1997), and by sequestering E47 which is needed by the proneural bHLHs to activate transcription (Sasai et al., 1992). The Id family also maintains neural progenitors by sequestering E proteins which are required by the proneural bHLHs (Norton et al., 2000). In addition, the Id family interacts with Rb and promotes cell cycle progression by preventing the inhibition of the E2Fs by Rb (Iavarone et al., 1994; Lasorella et al., 1996; Toma et al., 2000).

The proneural bHLHs, which include Mash1, Ngn1 and Ngn2, are expressed in the telencephalon and are responsible for the initiation of neurogenesis (Guillemot et al., 1993; Lo et al., 1991; Sommer et al., 1996;). They are transcriptional activators that require forming a heterodimer with the ubiquitously expressed bHLH E47 to initiate transcription of genes and bind to E box consensus sites (CANNTG) (reviewed in Bertrand et al., 2002). Following expression of the proneural bHLHs, is the expression of bHLHs involved in terminal differentiation like NeuroD (Lee et al., 2000), NeuroD2 (Farah et al., 2000), and Math2 (NEX) (Bartholoma et al., 1994). These factors are expressed outside of the ventricular zone in the cortical plate where terminal differentiation takes place (Schwab et al., 1998).

The neurogenic phase is followed by a wave of astrocyte phase, which peaks around birth (reviewed in Mehler et al., 2002). The proneural bHLH factors have been shown to inhibit astrocyte differentiation and are down-regulated during this phase (Ma et al., 1996; Sommer et al., 1996). Surprisingly, the Hes family have been implicated in the astrocytic phase in other areas of the brain such as the hippocampus (Tanigaki et al., 2001) and in the retina (Furukawa et al., 2000) although it remains to be determined whether this is also true in the cortex.

The last phase of differentiation is the oligodendrocyte stage which peaks after birth (reviewed in Mehler et al., 2002). There are two bHLHs that have been shown to be important for this stage, Olig1 and Olig2, which are expressed in oligodendrocyte progenitors and are transcriptional repressors (Lu et al., 2000; Tekki-Kessaris et al., 2001).

### *Hes*

There are currently seven members of the Hes family (Hes1-7) which are homologs of the Hairy and Enhancer of Split genes first discovered in *Drosophila* (reviewed in Kageyama et al., 2005). The Hes family have a bHLH domain in their N-terminal region which make them members of the bHLH superfamily of transcription factors. Other shared domains of the Hes family include an orange domain, which is involved in mediating the specificity of protein interactions (Dawson et al., 1995; Castella et al., 2000), and a WRPW (trp-arg-pro-trp) motif located in the C-terminal end, which interacts with the Groucho/TLE corepressor protein (Paroush et al., 1994; Fisher et al., 1996; Grbavec et al., 1996).

The Hes family uses the basic region of the bHLH domain to interact with DNA, which preferentially binds N boxes made up of the following sequence (CACNAG) (Sasia et al., 1992). In contrast, most other bHLH family members usually bind E boxes (reviewed in Ross et al., 2003). This difference is due to a proline residue located in the middle of the Hes family basic region which is a unique characteristic conserved in both flies and mammals (Davis et al., 2001; Iso et al., 2003).

Hes1, Hes3, and Hes5 are all expressed in neural precursor cells (Hatakeyama et al., 2004; Kageyama et al., 2005). The expression of Hes1 and Hes3 appears before the expression of either Notch or Delta (Hatakeyama et al., 2004). This implies that early Hes1 expression is controlled by factors other than Notch pathway signalling. Notch and Delta expression appear at E8.5 in the murine ventricular zone and this coincides with Hes5 expression. After this time point both Hes1 and Hes5 have been shown to be Notch effectors (Ohtsuka et al., 1999). There is no evidence that Hes3 is controlled by Notch signalling at any time during cortical development (Nishimura et al., 1998). Hes6 is also expressed in the brain and is unique among the Hes family in that it actually promotes neuronal differentiation by forming a heterodimer with Hes1 and inhibiting its function (Bae et al., 2000). Hes6 expression is induced by the proneural genes (Koyano-Nakagawa et al., 2000).

Hes1 in particular, uses two mechanisms to inhibit the proneural genes like Mash1. The first mechanism is direct transcriptional repression whereby Hes forms a homodimer or heterodimer with other Hes family members or with members of the HERP family

(Leimeister et al., 2000; Iso et al., 2001b; Iso et al., 2003). This dimer binds to N boxes in the proneural gene promoter, and recruits the protein TLE/Groucho (Paroush et al., 1994; Fisher et al., 1996; Grbavec et al., 1996), which in turn recruits a repressor complex which includes histone deacetylase (Chen et al., 1999). The second mechanism of repression involves Hes forming a heterodimer with E47. Since E47 is a required to bind Mash1 to initiate transcription, Hes inhibits Mash1 by sequestering its binding partner (Sasai et al., 1992).

The murine Hes1 promoter and gene was characterized by Takebayashi et al. (1994). It is located on chromosome 16 at position 26. Interestingly the Hes1 promoter contains four N box sequences which bind Hes1. It was shown that Hes1 can regulate its own expression in a negative feedback loop (Takebayashi et al., 1994). Hirata et al. (2002) also showed that Hes1 mRNA and protein were found to have very short half lives, ~24hours and ~22 hours respectively and that this contributed to a two hour oscillatory expression cycle of Hes1. This oscillatory nature of Hes1 is thought to be important in several developmental systems such as somite segmentation (Hirata et al., 2002; Bessho et al., 2003), although it remains to be determined if there is a significant role for this property of Hes1 in cortical development.

### *The Notch Pathway in Cortical Development*

The importance of the Notch pathway in development has been underscored by the severe developmental phenotypes of mice and flies deficient for one or more members of the pathway.

Notch1 was the first notch pathway member to be knocked out in mice. Notch1-/- mouse embryos die by E11.5 (Conlon et al., 1995) and show an increase in neuronal markers such as Math 4A, NeuroD, and NSCL-1 (de la Pompa et al., 1997). Conditional Notch1 mutants were created using the flox- cre method, with cre driven off the nestin promoter to test the effect of deleting Notch1 in neural precursors. This resulted in premature neuronal differentiation (Yang et al., 2004). Notch1 was also deleted in the mouse telencephalon using cre driven off the Foxg-1 promoter. This also resulted in premature neuronal differentiation and progenitor depletion (Yoon et al., 2004).

Following up evidence from studies in Drosophila where the Hairy gene was shown to inhibit neurogenesis along with the expression pattern of Hes1 in the ventricular zone where the undifferentiated progenitors are located (Sasai et al., 1992), a retrovirus containing Hes1 was injected into the lateral ventricle of mice to determine if Hes1 inhibited neurogenesis in mammals (Ishibashi et al., 1994). They found that cells infected with the virus and persistently expressing Hes1 did not undergo neuronal differentiation or migrate away from the ventricular zone. This showed that Hes1 did indeed inhibit neurogenesis in the mammalian brain. The Hes1 knockout mouse generated by Ishibashi et al, (1995) later confirmed the findings of Hes1 as an inhibitor of neuronal differentiation as there was an increase in the expression of neuronal markers Mash1 and NSCL1. Hes1 -/- embryos die by the age of E14.5 from premature neuronal differentiation and from complications in neural tube closure (Ishibashi et al., 1995).

The self-renewal ability of Hes1-/- neural precursors was first assessed by Nakamura et al. (2000) using the neurosphere assay. They found that Hes1-/- neural precursors were decreased in number and had reduced self-renewal ability. Ohtsuka et al.

(2001) also assessed the self-renewal ability of Hes1-/- neural precursors using the neurosphere assay and in contrast to the previous study, found that there was no difference in the number or self-renewal ability in Hes1-/- neural precursors compared to wildtype. This difference was attributed to the differences in mouse strains used in the two experiments, which shows that this effect is strain dependent.

Ohtsuka et al (1999) showed that double Hes1: Hes5 knockout mice exhibit a more severe phenotype compared to Hes1-/- or Hes5 -/- mice. This indicated that Hes1 and Hes5 compensate for each other to some extent. Ohtsuka et al. (1999) also showed that both Hes1 and Hes5 were effectors of Notch signalling, by overexpressing a constitutively active form of Notch (NICD), which inhibits wildtype neural progenitors from differentiating. When either Hes1 or Hes5 were removed, NICD still inhibited differentiation, however, when both Hes1 and Hes5 were removed, over expressed NICD could no longer inhibit differentiation. This showed that either Hes1 or Hes5 are required for Notch activity in neural precursors.

Mice triple mutant for Hes1, Hes3 and Hes5 show severe neural defects (Hatakeyama et al., 2004). By E10.5 all radial glia are differentiated into early born neurons at the expense of all later born cells types and embryos die at this time. With the loss of radial glia, the basal lamina and apical intercellular junctional complex, which mark the inner and outer boundaries of the neural tube, are lost, resulting in cells becoming scattered into the lumen (Hatakeyama et al., 2004). These results show that the Hes genes are essential for the maintenance of radial glia and for the structural integrity of the nervous system. It is interesting to note that in triple Hes mutant brains neuroepithelial cells are correctly formed but only after E8.5 begin to differentiate and prematurely disappear. This suggests that formation of neuroepithelial cells is Hes independent, but the maintenance of these cells becomes Hes dependent after E8.5 (Hatakeyama et al., 2004). These findings are consistent with other studies that have shown the Notch pathway to be essential for the maintenance of neural stem cells but not the formation. For example, in the absence of Notch 1, RBP-Jk, or presenilin1 neural stem cells were prematurely depleted (Hitoshi et al., 2002b). The self renewal ability of neural precursors missing Notch1, RBP-Jk, presenilin1, or presenilin2 was assessed using the neurosphere assay. At E10.5 there were very few neurospheres formed in Notch1-/- neural precursor cultures and no neurospheres formed at E8.5 in RBP-Jk-/- neural precursor cultures. Presenilin1-/- and Presenilin 2-/- neural precursor cultures had significantly fewer primary and secondary neurospheres forming compared to wildtype (Hitoshi et al., 2002b). These results showed that the self-renewal ability is decreased in neural precursors where Notch pathway members are absent.

## III. The Rb Family of Pocket Proteins (General)

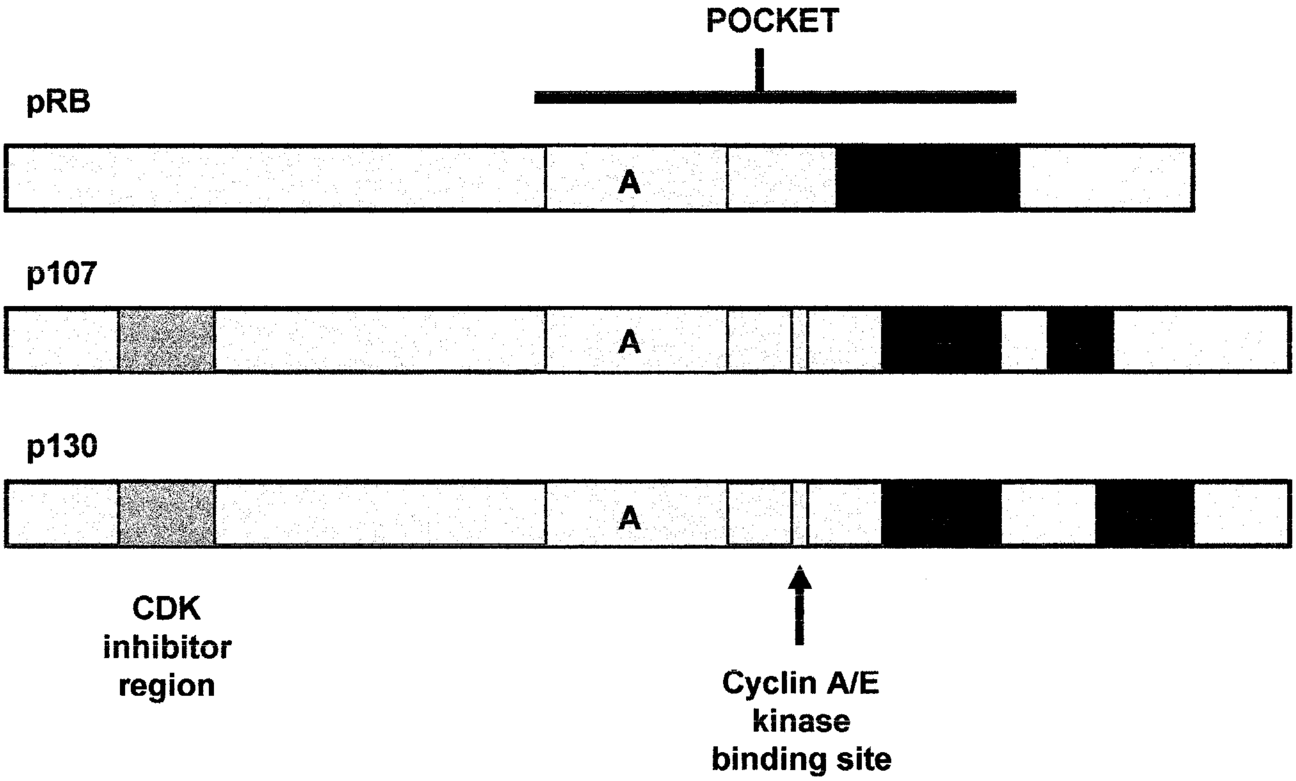
There are three members of the pocket protein family, Retinoblastoma susceptibility gene (Rb), p107 and p130, which function as cell cycle regulators (reviewed in Cobrinik et al., 2005). Rb was the first member to be discovered as the cause of a rare childhood form of retinal cancer, retinoblastoma, which arises when the Rb gene is mutated (Friend et al., 1986; Lee et al., 1987). Rb has subsequently been found to be mutated or disabled in many types of cancers (Harbour et al., 1988; Lee et al., 1988; Horowitz et al., 1989) and therefore is considered to be a tumour suppressor.

### ***Structure and Function***

All three proteins are similar in terms of sequence and protein structure, with p107 and p130 being the most closely related (Classon et al., 2001). The defining feature of this family is the pocket domain, which is divided into two parts designated A and B (Figure 2). The pocket domain interacts with the E2F family of transcription factors as well as viral oncoproteins and is located in the c-terminal end (Ewen et al., 1992; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). This family also possesses a LXCXE sequence in their pocket domain that binds HDAC1, HDAC2 and BRG1, and a Nuclear Localization Signal in their c- terminal end (Dunaief et al., 1994; Brehm et al., 1998). Unlike Rb, P107 and p130 contain anextended linker sequence in the region separating the A and B portions of the pocket domains that includes a binding site for cyclin A/cdk2 and cyclin E/cdk2 (Faha et al., 1992; Lees et al., 1992). P107 and p130 also have sequences in their N-terminal through which they are thought to act as cyclin dependent kinase (CDK) inhibitors, although the function remains to be determined (Castano et al., 1998; Woo et al., 1997).

There is significant compensation between members of the pocket protein family (Cobrinik et al., 1996; Lee et al., 1996; Sage et al., 2000). This is evident from the phenotypes of animals that have more than one pocket protein deleted and exhibit a more severe phenotype in comparison to animals with only one pocket protein deleted. For example, double mutant Rb-/-; p107-/- and Rb-/-; p130 -/- die two days earlier (E12) than Rb-/- embryos (Lee et al., 1996; Sage et al., 2000). Rb-/- mutants die around E14 and show defects in the nervous system, skeletal muscle, and lens (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). In addition, p107-/- and P130-/- mice on non- BALB/cJ strains show no apparent phenotype (Lee et al., 1996; LeCouter et al., 1998a; LeCouter et al.,

Figure 2. **Structure of the Retinoblastoma Family of Pocket Proteins.** The pocket domain of all family members is divided into two regions designated A and B. The B portion of the pocket in P107 and p130 contains an insertion sequence. P107 and p130 possess a cyclin A/E kinase binding site in the spacer region between A and B, which is absent from Rb. P107 and p130 also contain a CDK inhibitor region in their N-terminal. Adapted from Classon et al., (2001).



Adapted from Classon et al., (2001)

1998b), however double null p107-/-; p130-/- mutants die shortly after birth and show severe bone development complications (Cobrinik et al., 1996). These double knockout experiments show that the different pocket proteins can compensate for one another to a certain extent.

The pocket proteins differ in both their expression patterns and phosphorylation state during the cell cycle. Rb is expressed throughout the cell cycle and is present in both dividing and quiescent cells. It becomes phosphorylated during the G1 phase and remains so until late mitosis. p107 is expressed primarily in actively cycling cells and is expressed at low levels in quiescent cells (Kiess et al., 1995). It also becomes phosphorylated as cells near the S phase of the cell cycle (Beijersbergen et al., 1995). In contrast, p130 expression is elevated as the cells enter a quiescent state or differentiate (Smith et al., 1996) and is expressed at low levels in actively dividing cells.

### *Cell Cycle Regulation*

The function of these proteins is to regulate cell cycle progression, and this is achieved mainly by regulating the expression of cell cycle progression genes through the E2F family of transcription factors. For example, they regulate the transition between G1 and S phases of the cell cycle through slightly different mechanisms (Classon et al., 2001). Rb preferentially associates with the activating E2Fs (E2F1-3a) and in a hypophosphorylated state binds to the transactivating domain of the E2Fs. This interaction prevents them from initiating transcription of genes that are involved in S phase progression. Once Rb becomes phosphorylated by CDK4 or CDK6, it dissociates from E2F, which is then free to initiate transcription (Buchkovich et al., 1989; Hinds et al., 1992;

Lundberg et al., 1998). P107 interacts preferentially with E2F4 and actively represses transcription by forming a repression complex at the promoter of E2F responsive genes. Like Rb, p107 phosphorylation by cyclin D1-CDK4 results in dissociation with E2F (Beijersbergen et al., 1995; Xiao et al., 1996). As the cell nears the end of the G1 phase, both Rb (Buchkovich et al., 1989) and p107 (Xiao et al., 1996) become phosphorylated and this results in transcriptional repression being removed from E2F responsive genes. In the absence of Rb, cells transition between the G1 and S phase more quickly (Herrera et al., 1996), and in the absence of p107 cells spend a decreased amount of time in the S phase (LeCouter et al., 1998b).

### *P107*

Each of the pocket proteins have overlapping functions and can compensate for one another to varying degrees, however they also have distinct roles. In addition to its role as a cell cycle regulator, P107 has been shown to be involved in several processes including stem cell self-renewal (Vanderluit et al., 2004), protein synthesis (Makris et al., 2002) and maintenance of genome stability (Chibazakura et al., 2004). The pocket proteins also have distinct expression patterns. In the developing brain for example, p107 is highly expressed in the ventricular zone where the neural stem cells and progenitors reside (Jiang et al., 1997; Vanderluit et al., 2004). This is consistent with previous findings that p107 is expressed in proliferating, cycling cells, but is down regulated during terminal differentiation (Kiess et al., 1995).

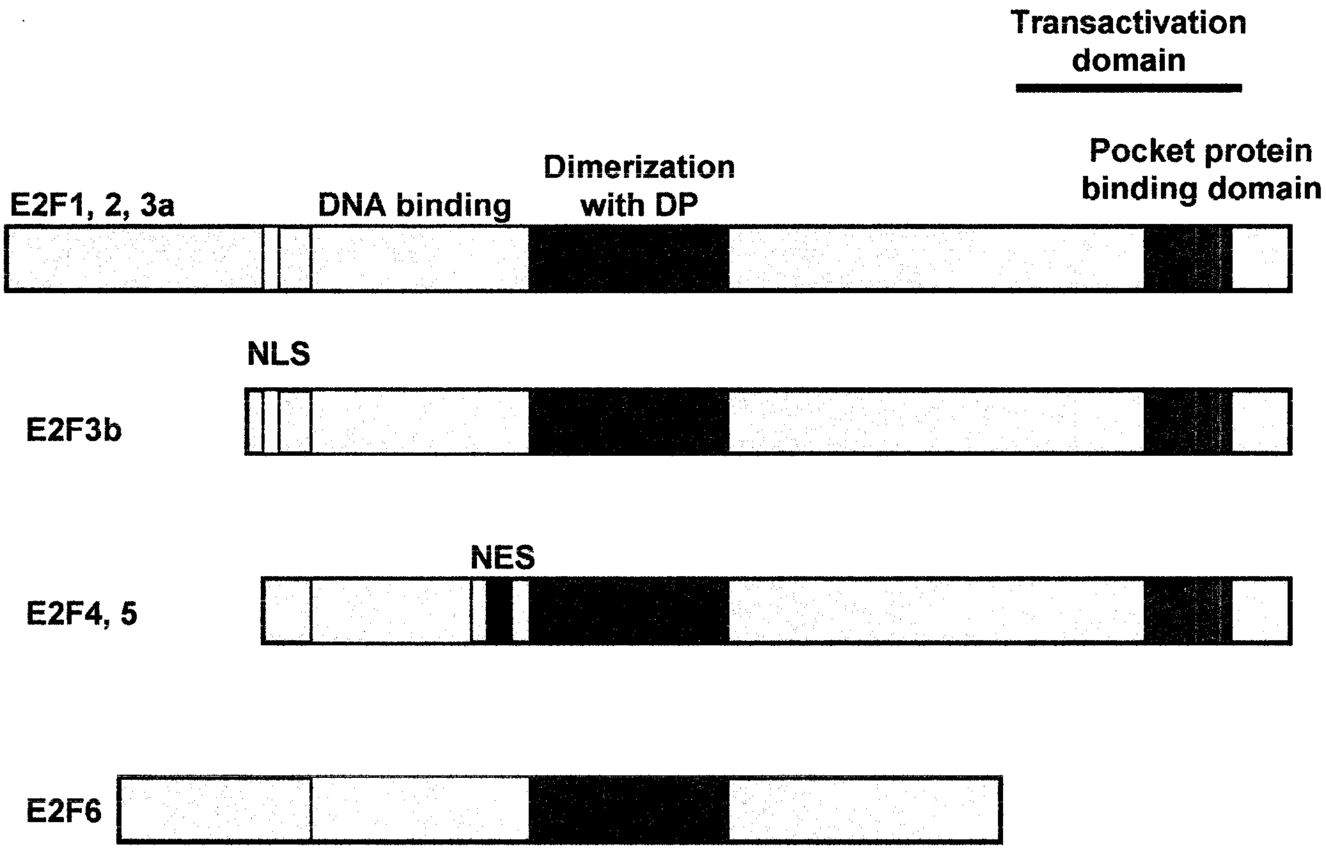
P107 deficient transgenic mice were generated independently by both Lee et al. (1996) and LeCouter et al (1998b). On a 129/Sv;C57BL/6J strain background p107-/- mice showed no apparent phenotype, and cells isolated from these mice did not have any cell cycle defects (Lee et al., 1996). In contrast, p107-/- pups on a BALB/cJ strain background showed decreased growth rate and muscle proliferation deficiencies. Fibroblasts isolated from these p107-/- embryos showed a two-fold decrease in cell cycle time (LeCouter et al., 1998b).

P107 has recently been found to negatively regulate neural stem cell self-renewal (Vanderluit et al., 2004). In p107-/- embryos and adults there is an increase in the number of neurosphere forming cells. This effect is specific to p107, and not Rb, as there was not found to be an increase in the number of neurosphere forming cells of Rb-/- neural precursor cultures compared to wildtype. In addition, removing Rb from p107 -/- neural precursors did not increase the number of neurosphere forming cells compared to p107-/- neural precursor cultures (Vanderluit et al., 2004). These experiments highlight the distinct role of p107 in regulating neural stem cell self-renewal.

### *The E2F family of Transcription Factors.*

The E2Fs are classified into three groups. The first group is the activating E2Fs, which include E2F1, E2F2, and E2F3a (Bagchi et al., 1991; Ivey-Hoyle et al., 1993; Lees et al., 1993). The second group is the repressive E2Fs comprised of E2F3b, E2F4, and E2F5 (Ginsberg et al., 1994; Hijmans et al., 1995; Leone et al., 2000). The remaining E2F family members, E2F6, E2F7, and E2F8, do not bind pocket proteins (Morkel et al., 1997; de Bruin et al., 2003; Di Stefano et al., 2003; Maiti et al., 2005). E2F1-5 form heterodimers with the DP family, which enables them to bind to DNA. The DP family includes three members, DP(1-3) (Trimarchi et al., 2002) (Figure 3).

Figure 3. **Structure of the E2F family of Transcription Factors .** Structural comparison of the first six E2F family members to be discovered which can all form dimers with DP. E2F1-3 contain a Nuclear Localization Signal (NLS) in contrast to E2F4-5 which contain a Nuclear Export Signal (NES). E2F1-5 possess a pocket protein binding site within the transactivation domain in their C-terminal. Adapted from Trimarchi et al., (2002).



Adapted from Trimarchi et al., (2002)

There are several differences between these groups stemming from their protein structure. The activating E2Fs contain a nuclear localization signal in the N-terminal region and are always found in the nucleus (Allen et al., 1997). In contrast, the repressive E2Fs contain a leucine/isoleucine-rich hydrophobic nuclear export signal and are mostly found in the cytoplasm (Gaubatz et al., 2001). They require binding to one of the pocket protein family members or DP2 to enter the nucleus. The Rb-family independent E2Fs lack the Rb family binding site in their C-terminal region, and repress transcription through Rb family independent mechanisms (Morkel et al., 1997; de Bruin et al., 2003; Di Stefano et al., 2003; Maiti et al., 2005).

The expression of the activating E2Fs (1-3a) is cell cycle dependent and starts in late G1 phase (Lees et al., 1993; Johnson et al., 1994; Neuman et al., 1994). They are responsible for initiating the transcription of several types of genes including cell cycle regulation genes such as pRB (Hamel et al., 1992), p107 (Zhu et al., 1995), cyclin E (Botz et al., 1996), cyclin A (Schulze et al., 1996), Cdc2 (Dalton et al., 1992), and Cdc 25A (Vigo et al., 1999), genes involved in DNA synthesis machinery including ORC1 (Asano et al., 1999) and Cdc6 (Ohtani et al., 1998), and genes encoding enzymes involved in biosynthesis of nucleotides such as thymidine kinase (Good et al., 1995), dihydrofolate reductase (Blake et al., 1989), and thymidylate synthetase (DeGregori et al., 1995). Over expression of the activating E2Fs can drive quiescent cells into the cell cycle (Johnson et al., 1993; Lukas et al.,1996). E2F3 was first characterized by Lees et al. (1993). It was later discovered that a second shorter transcript was originating from the E2F3 gene off a promoter located in the first intron of the originally described gene, which is now referred to as E2F3a (Leone et al., 2000). This second shorter transcript encodes a protein referred to as E2F3b and is identical to E2F3a except for lack of the N-terminal domain present in E2F3a. E2F3a is considered to be an activating E2F, and E2F3b is a repressive E2F (Leone et al., 2000).

### *Repressive E2Fs*

The expression of the repressive E2Fs (3b-5) is not cell cycle dependent. They are expressed constitutively throughout the cell cycle (Cobrinik et al., 2005). E2F4 interacts with all of the pocket proteins (Attwoll et al., 2004) whereas E2F5 mainly interacts with p130 (Hijmans., 1995). The pocket protein-E2F repressor complex actively represses transcription by binding to E2F sites on the promoter and recruiting proteins that are involved in chromatin remodelling such as histone deacetylases (HDACs) (Ferreira et al., 1998), brahma (BRM) (Strobeck et al., 2002), and brahma-related gene1 (BRG1) (Dunaief et al., 1994). The HDACs function by removing acetyl groups from histones H3 and H4. This results in the chromatin condensing and preventing other transcription factors from accessing the DNA and initiating transcription (Kornberg et al.,1995; Wolffe et al., 1999).

### *The Interaction of the p107/E2F and the Notch Pathway*

Consistent with its role as a regulator of neural stem cell self-renewal, p107 has also been shown to interact with the Notch pathway (Vanderluit et al., 2004), which is also involved in stem cell self-renewal (Nakamura et al., 2000; Ohtsuka et al., 2001; Hitoshi et al., 2002b). Vanderluit et al. (2004) showed an increase in the protein levels of active Notch (NICD) in p107 -/- neurospheres, demonstrating an increase in Notch pathway activity in the absence of p107. They also showed that when p107 is overexpressed in a HEK 293 cell line, the protein levels of both full length and activated Notch are decreased. In addition several E2F binding sites were found on the Notch1 gene and promoter, with p107 binding activity demonstrated at an E2F binding site in intron1 using chromatin immunoprecipitation (Vanderluit et al., 2004). Since the Notch pathway has previously been showed to be involved in stem cell self-renewal, these results suggest that p107 is regulating the neural stem cell pool through interactions with the Notch pathway.

## IV. Research Objectives

Based on previous findings from our group that p107 negatively regulates neural precursor self-renewal and that Notch activity is elevated in homozygous p107 null (p107-/-) neural precursors (Vanderluit et al., 2004), ***we hypothesized that p107 negatively regulates neural stem cell self-renewal by directly repressing Hes1 transcription through interactions with E2F.***

To address this hypothesis there are two objectives.

1) Examine the mechanism by which p107 modulates the Notch1-Hes1 signalling pathway. To investigate this, we first examined the levels of Hes1 transcript and protein levels in p107-/- brains and neural precursors using *in situ* hybridization and western blot analysis respectively. We then examined the Hes1 promoter for E2F binding sites and finally used the luciferase reporter assay to determine if the Hes1 promoter was responsive to either p107 or its regulatory targets E2F3 and E2F4.

2) Determine if the elevated Hes1 expression is responsible for the increased self-renewal of neural precursors in p107-/- embryos. To investigate this, we crossed heterozygous mutant p107+/- and heterozygous mutant Hes1+/- mice to produce double null p107-/-: Hes1-/-, and examined the neural precursor cell number using the primary neurosphere assay. If the increased number of neural precursor cells in p107-/- mice is due to p107 enhanced levels of Notch1 and Hes1, then deletion of a downstream member of that pathway, such as Hes1, should restore the number of neurosphere forming cells to wildtype levels.

## Materials and Methods

### *Transgenic Mice*

Germline p107 null mice, obtained from Dr. Michael Rudnicki, were generated previously by LeCouter et al., (1998b) and maintained on a mixed SV-129 and C57Bl/6 background. Germline Hes1 null mice on a C57Bl/6 background strain, were originally generated by Ishibashi et al. (1995). To generate Hes1/p107 deficient embryos, heterozygous (Hes1+/-) mice were interbred with p107+/- mice to produce mice the following genotypes: Hes1-/-:p107-/-, Hes1+/-:p107-/-, Hes1+/-:p107+/+, Hes1+/-:p107+/-, Hes1-/-, p107-/- and wild type mice. For embryonic time points, the time of plug identification was counted as day 0.5. All experiments were approved by the University of Ottawa's Animal Care ethics committee adhering to the Guidelines of the Canadian Council on Animal Care.

### *DNA Isolation*

DNA isolation was performed according to the protocol of Laird et al. (1991). For genotyping, tail clippings or embryonic tissue was digested in 500 μL of DNA lysis buffer (100 mM Tris, 5 mM EDTA, 0.2 % SDS, 200 mM NaCl, ddH$_2$O, pH 8.0) and 10 ng of proteinase K (Gibco, 25530-031) overnight at 55 °C. 500 μL of phenol:chloroform was added to each sample, shaken and incubated for 10 mins, then centrifuged for 5 mins at 12 000 rpm at room temperature. Upper aqueous phase was transferred to a fresh tube and 500 μL of cold isopropanol was added and rocked for 2 mins. Samples were centrifuged for 5 mins at 12 000 rpm. Liquid was aspirated and pellet was washed with 1 mL of 70 %

ethanol, then dried for 5 mins at 55 °C, and dissolved in 50 µL of TE (10 mM Tris, 1.0 mM EDTA, ddH2O, pH 8.0).

### *Polymerase Chain Reaction (PCR)*

Genotyping of p107 deficient and Hes1 deficient mice was carried out by PCR according to the protocols of LeCouter et al. (1998b) and Ishibashi et al. (1995) respectively. The primer sequences are shown in Table 1, along with corresponding annealing temperatures. The following PCR conditions were used: 94 °C - 5 mins, 92 °C - 1 min, annealing temperature (Table1) - 1 min, 72 °C - 1.5 mins, 72 °C - 5 mins (31 cycles). All PCR products were electrophoresed through a 1 % agarose gel containing ethidium bromide (Sigma, E1510-10ML) and visualized with an AlphaImager 2200 (Alpha Innotech Corporation).

### *Primary Cultures of Neurospheres*

Neurospheres were cultured according to the protocols of Tropepe et al. (1999). Pregnant mice were sacrificed with a lethal dose (900 mg/ kg body weight) of sodium pentobarbitol and embryos at either embryonic day (E) 10.5 or 13.5 were removed and washed in 1 X Phosphate Buffered Saline (PBS) (8g/L NaCl, 0.2g/L KCl, 1.44g/L $Na_2HPO_4$, 0.42g/L $KH_2PO_4$, ddH2O, pH 7.4) then transferred to cold 1 X Hank's buffered saline (HBSS), pH 7-7.3 (Gibco, 14185-052). Under a dissecting microscope (Zeiss, Stemi 2000-C) the ganglionic eminences of E13.5 embryos or the entire telencephalon of E 10.5 embryos were dissected and placed into 1 mL of stem cell media (2 mM L-glutamine, 6 g/L D-glucose, 25 µg/L insulin, 10 µg/L apotransferine, penstrep, 0.02 µM progesterone, 9.6 mg/L

Table 1: Primer sequence and corresponding annealing temperature.

| **Primer Name** | **Primer Sequence** | **Annealing Temperature (°C)** |
|---|---|---|
| P107 FORWARD | 5'-TCG TGA GCG GAT AGA AAG -3' | 48 or 58 |
| P107 REVERSE | 5'-GTG TCC AGC AGA AGT TA-3' | 58 |
| P107 NEO | 5'- CCG CTT CCA TTG CTC AGC GG-3' | 48 |
| HES1 FORWARD | 5'-ATA TAT AGA GGG GGA CAG GGC CTG CGG ATC-3' | 72 |
| HES1 RWT | 5'-CGC AGG TAC TGT CTT AGG TTT CTG TGC TCA GAG GCC-3' | 72 |
| HES1 RKO | 5'-CGG TTC CAT TGC TCA GCG GTG CTG TCC ATC-3' | 72 |
| ChIP Hes1 E2F BS1 FORWARD | 5'-CGG TGC CGC GTG TCT CTT CCT-3' | 54 |
| ChIP Hes1 E2F BS1 REVERSE | 5'-TTG GTT TGT CCG GTG TCG TGT TGA -3' | 54 |
| ChIP Hes1 E2F BS2 FORWARD | 5'-ATA AAA TTC TCT GGG GAC TGA -3' | 54 |
| ChIP Hes1 E2F BS2 REVERSE | 5'-ATG ATA GGC TTT GAT GAC TGC -3' | 54 |
| ChIP Hes1 E2F BS3 FORWARD | -CTT GCC CGC CGT CTA TCC GTA T-3' | 56 |
| ChIP Hes1 E2F BS3 REVERSE | 5'-TCC CGC TCG AAC TCT GTA TGT -3' | 56 |

putrescine, 0.03 μM selenium, 12.5 μg/L fungizone, 20 μg/L bFGF, 2 mg/L heparin, DMEM-F12). A portion of tissue was taken for genotyping. Ganglionic eminence or telencephalon tissue was dissociated into a single cell suspension by trituration, cells were counted using a hemacytometer, and cultured in stem cell media at the appropriate cell density and incubated in a $CO_2$ Water Jacketed Incubator (Forma Scientific) at 37 °C and 5% $CO_2$. For chromatin immunoprecipitation and western blot analysis cells were plated in 60 mm dishes at 1 million cells/dish. For primary neurosphere assay cells were plated at 5000 cells/well in a 24 well dish (Nunc). Neural stem cells were split into twice the volume of media four days after culturing and passaged seven days after culturing. To passage cells, neurospheres were triturated to a single cell suspension and plated at a density of 1 million cells/ 60mm dish. For primary neurosphere assay neurospheres were counted seven days after culturing (Tropepe et al., 1999).

### *Maintenance of Human Embryonic Kidney (HEK) 293 cells*

HEK 293 cells, originally generated by Graham et al. (1977), were obtained from American Type Culture Collection, Manassas, VA, USA and were cultured in Dulbecco's Modification of Eagle's Medium 1X (DMEM) (MultiCell, 319-015-CL) supplemented with heat inactivated fetal bovine serum (Sigma, 080-150) and penstrep (Invitrogen, 15410-122). Cells were passaged once they reached between 80% - 90 % confluency.

### *Tissue Fixation and Cryoprotection*

Pregnant mice were sacrificed by injection of a lethal dose of sodium pentobarbitol (900 mg/ kg body weight). E14.5 embryos were removed and washed in 1 X PBS followed by fixation in 4 % paraformaldehyde (PFA)/ 0.1 M phosphate buffer pH 7.4 for two days at 4 °C. Fixed tissue was washed three times in PBS and then placed in each of the following increasing concentrations of sucrose solutions for one day at 4 °C ; 12 %, 16 % and 18 % sucrose/ 0.1 M phosphate buffer. Tissue was then embedded in OCT (TissueTek, 4583), frozen on liquid nitrogen, and sectioned using a cryostat (Microm, HM500) into 14 µm sections at -20 °C, which were then placed on Superfrost slides (Fisher, 12-550-15) and stored in -80 °C.

### *Chromatin Immunoprecipitation*

Chromatin immunoprecipitation was performed using a Chromatin Immunoprecipitation Assay Kit (Upstate, 17-295) according to manufacturer's instructions. Neurospheres were cultured for four days, then triturated into a single cell suspension. 1.5 million cells were incubated in 1 % formaldehyde for 15 minutes at 37 °C, then centrifuged at 2000 rpm at 4 °C for 5 mins. Supernatant was removed and cells were washed with cold 1 X PBS containing protease inhibitors (PMSF, aprotinin, and pepstatin) then incubated in SDS Lysis Buffer (Upstate, 20-163) for 10 mins on ice. Samples were sonicated using a 60 Sonic Dismembrator (Fisher Scientific) on setting three for 10 seconds. This was repeated three times between 1 min incubations on ice. Samples were centrifuged at 13 000 rpm for 10 mins at 4 °C, supernatant was transferred to a fresh tube and diluted with ChiP Dilution Buffer (Upstate, 20-153). 70 µL of Salmon Sperm DNA/Protein A Agarose-50% slurry

(Upstate, 16-157) was added to the ChIP DNA samples, which were then incubated at 4 °C for 1 hour and centrifuged at 800 rpm for 5 mins at 4 °C. Supernatant was transferred to a fresh 1.5 mL ependorf tube, 4 µL of antibody directed against either E2F3 (Santa Cruz Biotechnology, SC-878) or E2F4 (Chemicon International, MAB88947) was added and incubated for 16 hrs at 4 °C while rocking. 60 µL of Salmon Sperm DNA/Protein A agarose-50 % slurry was added and incubated at 4 °C for 1 hour. Samples were centrifuged at 800 rpm at 4 °C for 2 mins. Supernatant was removed and pellet was sequentially washed with each of the following solutions for 5 mins at 4 °C ; 1) Low Salt Immune Complex Wash Buffer (Upstate, 20-154) 2) High Salt Immune Complex Wash Buffer (Upstate, 20-155) 3) LiCl Immune Complex Wash Buffer (Upstate, 20-156) and 4) 2 washes of 1 X TE. 0.1 M NaHCO3 with 1 % SDS was added to all ChIP and Input control samples, followed by incubation at room temperature for 15 mins. Samples were centrifuged at 800 rpm for 3 mins at 20 °C, and supernatant was transferred to a fresh 1.5 mL tube. 20 µL of 5 M NaCl was added to all samples and incubated for 4 hours at 65 °C. DNA was recovered and purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). PCR was used with primers in Table 1 to detect the presence of immunoprecipitated Hes1 promoter DNA.

### *Western Blot Analysis*

Western blot analysis was performed according to protocol previously described in Ferguson et al., (2000). Proteins were extracted from neurospheres with lysis buffer (50 mM HEPES, 250 mM KCl, 0.1 M EDTA, 0.1 M EGTA, 10% glycerol, 0.1% NP-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 g/mL aprotinin, 2 g/mL leupeptin, 0.4 mM sodium vanadate, pH 7.8). Briefly, samples were incubated on ice for 15 min in lysis buffer, then centrifuged for 5 min at 14000rpm at 4°C. Supernatant was transferred to a new tube and an aliquot was used for quantification using the Bio-Rad Assay. Proteins were boiled for 5 min and were separated on a 10% polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane, and incubated with blocking agent [5% skim milk in Tris-buffered saline/Tween20 (TPBS)] for one hour with rocking, followed by one 5 min wash with TPBS (100 mM $Na_2HPO_4$, 100 mM $NaH_2PO_4$, 0.5 N NaCl, 0.1% Tween 20). Membranes were incubated with Hes1 antibody (Ito et al., 2000) at a concentration of 1:1500 in 5% skim milk with TPBS overnight at 4 °C with rocking, then washed twice for 5 min in TPBS followed by incubation with secondary antibody anti-rabbit HRP at a dilution of 1:3000 (Bio Rad, 170-6515) in 2% skim milk in TPBS for 45 min at RT. Blots were developed according to manufacturer's instructions by chemiluminescence (ECL, Amersham Pharmacia Biotech). Kodak Developer - Kodax X-OMAT 200A Processor.

### ***Luciferase Reporter Assay***

Luciferase reporter assay was performed according to the protocol of Charest et al., (1993). The Hes1 promoter (~1500 bp), including all three putative E2F binding sites was isolated from wildtype murine DNA by PCR using the primers:

5'-CGCGGCGGCAATAAAACATC-3' and

5'-GATGAGTGCACAGGGGGAGAAAGACCTC-3', and inserted in the pGL3-Basic vector (Promega). HEK 293 cells were transfected using the calcium phosphate transfection method (Charest et al., 1993). Cells were split 24 hours prior to transfection and were at a confluency between 40 - 60 % at time of transfection. For each transfection reaction the following were added; 500 μL of 0.25 M $CaCl_2$, 2 μg of pGK-Lac Z, 10 μg of pGL3-Basic-Hes1-promoter vector and combinations of the following of expression vectors at 3-10 μg; Rb (pGK-RB), p107 (pCMV-p107), E2F3 (pCMV-E2F3), or E2F4 (pCMV-E2F4). The appropriate amount of Adllox was added to make the total amount of DNA equivalent in all reactions. Samples were vortexed while 500 μL of phosphate solution (16 g/L NaCl, 0.74 g/L KCl, 2 g/L dextrose, 10 g/L HEPES, 1.5 mM $Na_2HPO_4$, pH 7.05) was added drop wise. Media was aspirated out of 100 mm dish containing HEK 293 cells and prepared transfection solution was pipetted directly over cells and allowed to incubate for 10 mins. 10 mL of DMEM media was added and plates were incubated for 16 hours at 37 °C. Reaction was stopped by replacing media with 10 mL of Hepes media (40 mM HEPES in DMEM) and incubating cells for 4 hours at 37 °C followed by replating cells into three wells of a six well dish, which were incubated overnight at 37 °C.

Next day cells were lysed and harvested in 200 μL of 1 X Reporter Lysis Buffer (Promega, E397A). Cells were scraped and transferred to a 1.5 mL ependorf tube and incubated at -80 °C for at least one hour. Samples were then vortexed, and centrifuged at 12 200 g for 2 mins at 4 °C and supernatant was transferred to fresh tube. MUGs assay was performed to standardize the transfection reaction by determining the rate at which B-galactosidase converts 4-methylumbelliferyl-D-galactoside (MUG) (Sigma-Aldrich) to methylumbelliferone. 30 μL of MUGS substrate solution (0.3 mM MUG, 15 mM Tris-HCl, pH 8.8) was added to 10 - 20 μL of sample and incubated at 37 °C for 30 mins. 50 μL of MUGS Stop solution (300 mM glycine, 15 mM EDTA, pH 11.2) was added and samples were transferred to cuvettes (VWR International, 58017-875) containing 2 mL of Z-Buffer (60 nM $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$, $ddH_2O$ ), and incubated in the dark for 20 mins. A fluorescence reading was taken with a Perkin Elmer LS50 luminescence spectrofluorometer at 350 nm excitation and 450 nm emission settings.

Luciferase Assay was performed by adding 35 µL of sample to glass tubes (Sarstedt, 55.484). In the dark, Luciferin (Molecular Probes, L-2916) was added to Luciferase Buffer (20 mM tricine, 2.67 mM MgSO4, 1.07 mM $(MgCO_3)_4Mg(OH_2)5H_2O$, 0.1 mM EDTA, 33.3 mM DTT, 270 µM CoA, 530 µM ATP, pH 7.8). 100 µL of this solution was added to samples immediately before taking a reading with a BioOrbit 11250 luminometer.

### *In Situ Hybridization*

Non-radioactive *in situ* hibridization was carried out according to the protocol of Wallace et al. (1999). The following digoxigenin (DIG) labelled riboprobes were used; Hes1 antisense and sense (Tomita et al., 1996), Hes5 antisense and sense (Ishibashi et al., 1995; Ishii et al., 1997), Notch1 antisense and sense (Lindsell et al., 1995). Riboprobes were purified with LiCl ethanol precipitation. 2.5 µL of 4 M LiCl and 75 µL of prechilled ethanol was added to riboprobes, mixed well, and incubated at -80 °C for 30 minutes. Samples were centrifuged at 13 000 g for 15 mins at 4 °C. Ethanol was decanted and pellet was washed carefully with 50 µL of cold 70 % ethanol. Samples were centrifuged at 13 000 g for 15 mins. Ethanol was decanted and pellet was air dried for 10 mins, then dissolved in 50 µL of DEPC water or TE-buffer and stored in -80 °C.

Probes were diluted 1:500 to 1:2000 with hybridization buffer (1 X salt, deionized formamamide, 10 % dextran sulfate, 1mg/mL rRNA, 1 X Denhardt's, $ddH_20$), and denatured at 70 °C for 10 mins. 100 to 150 µL of probe was added to each slide and covered with a cover slip. Slides were incubated overnight at 65 °C in a slide box lined with Whatman paper soaked with 50% Formamide/10 X salt solution. Next day slides were washed several times with Wash Buffer (1 X SSC, 50 % formamide, 0.1 % Tween-20, $ddH_20$) at 65 °C, followed by two washes with 1X MABT (0.5 M Maleic acid, NaOH, 0.75 M NaCl, 0.5 % Tween 20) for 30 mins at room temperature while rocking.

Sections on slides were outlined with hydrophobic pen (DAKO, S2002) and blocking solution (20 % heat-inactivated sheep serum/ 2 % blocking reagent in 1 X MABT) was added and incubated in a humidified slide box for 1 hour at room temperature. Blocking solution was removed and 300 µL of anti-DIG antibody (BM, 1093274) was added. Slides were covered with coverslips then incubated overnight at room temperature.

Next day slides were washed five times with 1 X MABT for 20 mins at room temperature, followed by two washes with Pre Stain solution (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9-9.5, 0.1 % Tween-20) for 10 mins at room temperature. Slides were then incubated in Stain solution (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9-9.5, 0.1 % Tween-20, 10% PVA, 4.5 µL/mL NBT, 3.5 µL/mL of BCIP, $ddH_2O$) at room temperature for 4 hours. Stain reaction was stopped by washing slides several times with 1 X PBS for 10 mins with rocking at room temperature and coverslips were mounted in 80 µL of (1:1) glycerol/PBS and sealed with clear nail polish. Slides were stored at 4 °C and examined with a Ziess Axioskop 2 flourescence microscope. Pictures were taken with a Sony Power HAD 3CCD camera and Northern Eclipse software.

### ***Statistical analysis***

Error for values is expressed in standard error of the mean (SEM). Significance for both the luciferase assay and the neurosphere assay experiments was assessed using analysis of variance (ANOVA) and with a post hoc Tukey test. Statistical significance was set at $P < 0.05$.

## Results

### *Hes1 transcript and protein levels are elevated in p107-/- brains.*

Our group has shown that p107 negatively regulates neural precursor number and self-renewal in both embryos and adults. We also found an interaction between p107 and the Notch signalling pathway (Vanderluit et al., 2004). Previous studies have shown that the Notch pathway is required for neural stem cell self-renewal (Hitoshi et al., 2002b; Nakamura et al., 2000). Consistent with this, neural precursor cells in p107-/- mice have higher levels of Notch1 transcript compared to their wildtype littermate controls (Vanderluit et al., 2004) (Figure 4). Activated Notch1 upregulates expression of the bHLH transcription factors Hes1 and Hes5, which act to keep cells in the cell cycle by inhibiting neural differentiation genes (Ohtsuka et al., 1999). Since we have previously shown elevated levels of Notch1 in p107-/- mice, we asked if Hes1 and Hes5, effectors of the Notch pathway, were also upregulated. To answer this question, we first performed *in situ* hybridization on brain sections from p107 -/- and wildtype littermate embryos at E14.5 and E16.5. Sections were incubated with digoxigenin labelled antisense riboprobes for Hes1 and Hes5, with sense probes used as controls. Examination of *in situ* hybridization revealed that Hes1 transcript was upregulated in p107-/- brains (Figure 5). A similar result was observed in three separate embyros. The upregulation of Hes1 was found along the ventricular zone in p107-/- brains where p107 is normally expressed during development (Jiang et al., 1997; Vanderluit et al., 2004). Hes5 is also a Notch effector expressed in the ventricular zone during development (Akazawa et al., 1992). P107 -/- and wildtype sections were incubated with Hes5 antisense and Hes5 sense control riboprobes. No differences

Figure 4. **Notch1 transcript is upregulated in p107 -/- brains.** *In situ* hybridization was performed on brain sections from wildtype (A, C) and p107-/- (B, D) littermate embryos at E14.5 on coronal sections through the telencephalon. Sections were incubated with digoxigenin labelled Notch1 antisense riboprobe (A, B). Notch1 sense riboprobe was used as a control (C, D). Ventricular zone is labelled by VZ. Scale bar = 500 μm. (n = 3)

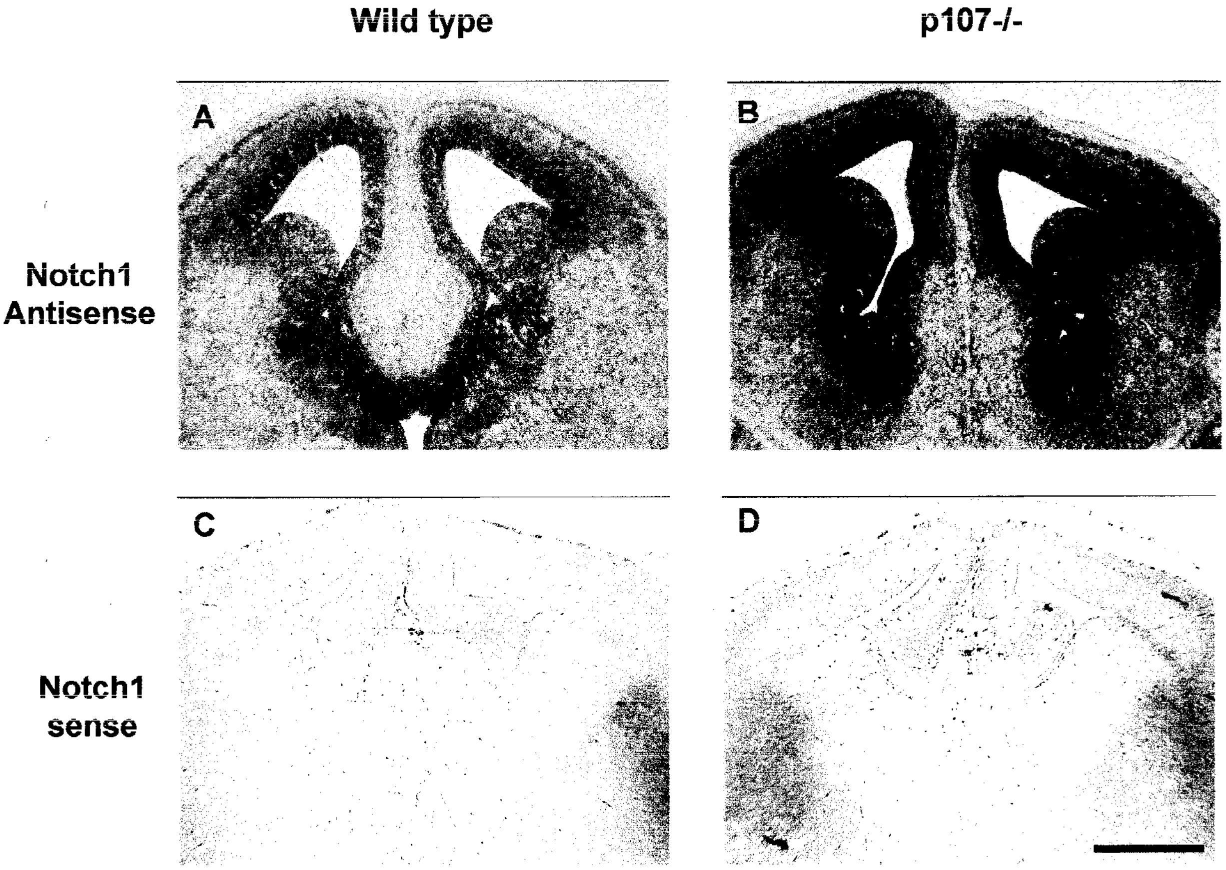
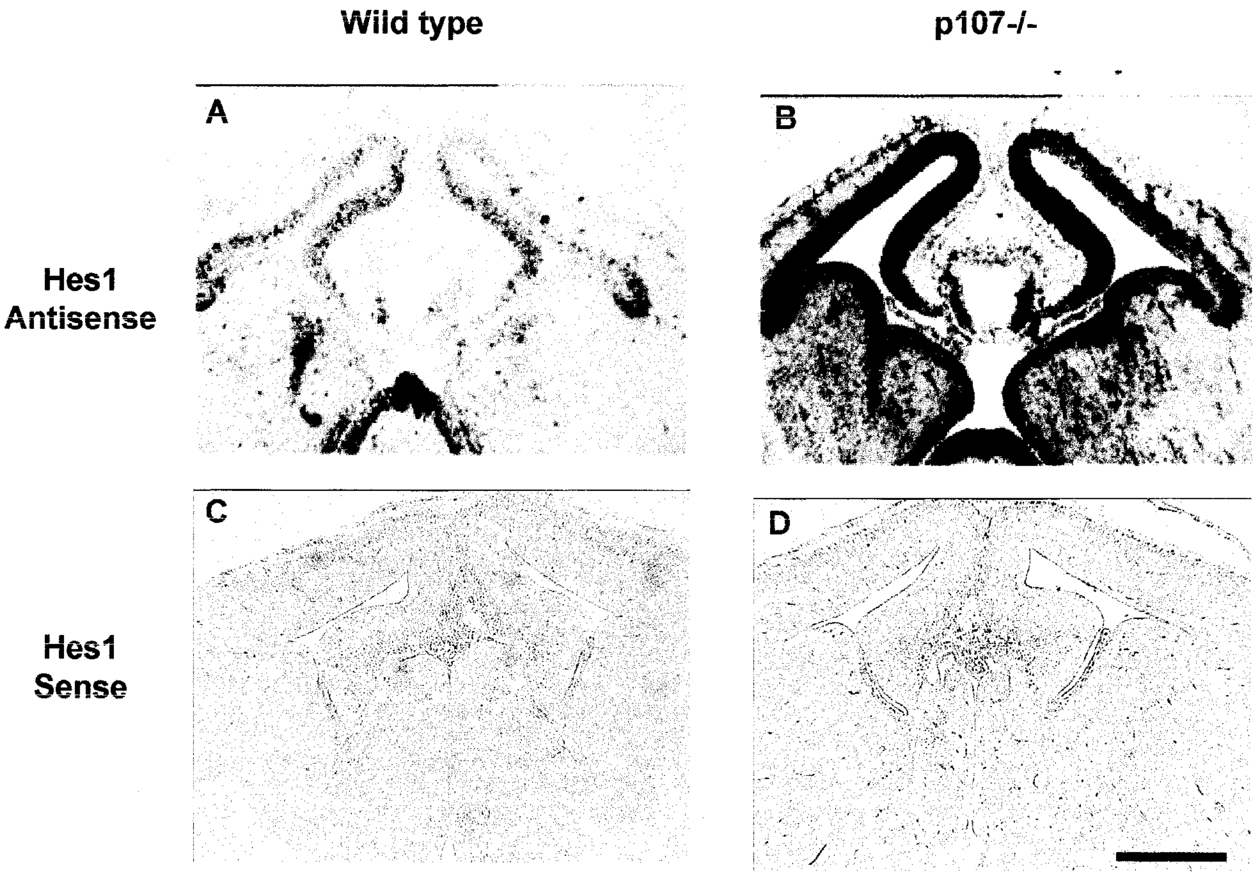

Wild type
p107-/-
Notch1
Antisense
Notch1
sense
A
B
C
D

Figure 5. **Upregulation of Hes1 transcript in E14.5 p107-/- brains.** *In situ* hybridization was performed on coronal sections through the telencephalon from wildtype (A, C) and p107-/- (B, D) littermate embryos at E14.5. Sections were incubated with digoxigenin labelled Hes1 antisense riboprobe (A, B). Hes1 sense riboprobe was used as a control (C, D). Scale bar = 500 μm. (n = 3)


Wild type
p107-/-
Hes1
Antisense
Hes1
Sense
A
B
C
D

were detected for Hes5 transcript levels in p107 -/- and wildtype embryos during development (Figure 6,7). Thus, the results of *in situ* hybridization revealed that in p107-/- brains the transcript levels of both Notch1 and Hes1 are upregulated and that there is no difference in Hes5 transcript.

We next asked whether protein levels of Hes1 were also upregulated in p107-/- neural precursor cells. Since no difference was detected for Hes5 transcript, only Hes1 protein was examined. Neurospheres were cultured from p107-/- brains and their wildtype littermates and protein was extracted for western blot analysis. Blots were probed with a Hes1 primary antibody obtained from Hitoshi Kitamura (Ito et al., 2000) and used at a dilution of 1:1500. The results of our studies revealed that Hes1 protein levels were also upregulated in p107-/- neurospheres relative to neurospheres from wildtype littermates (Figure 8).

These results show that the transcript levels of both Notch1 and its downstream effector Hes1 are upregulated in p107-/- neural precursor cells. This upregulation is specific since it was only seen with Hes1 and not Hes5. In addition, western analysis revealed that the protein levels of Hes1 were also upregulated. Our results examine the expression of members of the Notch pathway and suggest that p107 might be regulating Notch signalling during development and that the regulation is specific to Hes1. To investigate this possibility, we asked if p107 directly regulates Notch1 and Hes1 transcription through E2F sites in their promoters.

Figure 6. **Hes5 expression in wildtype and p107 deficient embryos.** *In situ* hybridization was performed on brain sections from wildtype (A, C) and p107-/- (B, D) at E14.5. Sections were incubated with digoxigenin labelled Hes5 antisense riboprobe (A, B). Hes5 sense riboprobe was used as a control (C, D). Scale bar = 500 µm. No difference was detected in Hes5 transcript levels between p107-/- and wildtype embryos at E14.5. (n = 3)

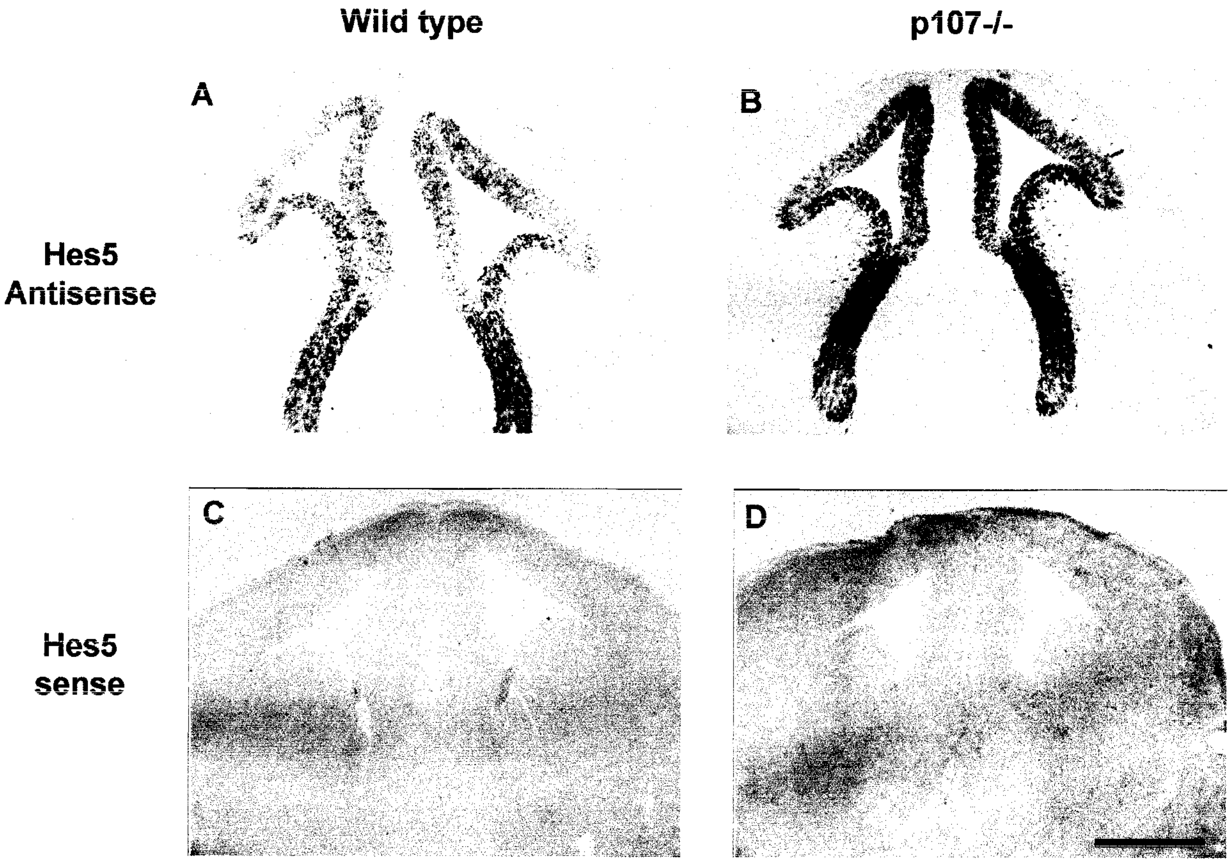

Wild type
p107-/-
A
B
Hes5
Antisense
C
D
Hes5
sense

Figure 7. **Hes5 expression in wildtype and p107 deficient embryos.** *In situ* hybridization was preformed on brain sections from wildtype (A, C) and p107-/- (B, D) at E16.5. Sections were incubated with digoxigenin labelled Hes5 antisense riboprobe (A, B). Hes5 sense riboprobe was used as a control (C, D). Scale bar = 500 μm. No difference was detected in Hes5 transcript levels between p107-/- and wildtype embryos at E16.5. (n = 3)

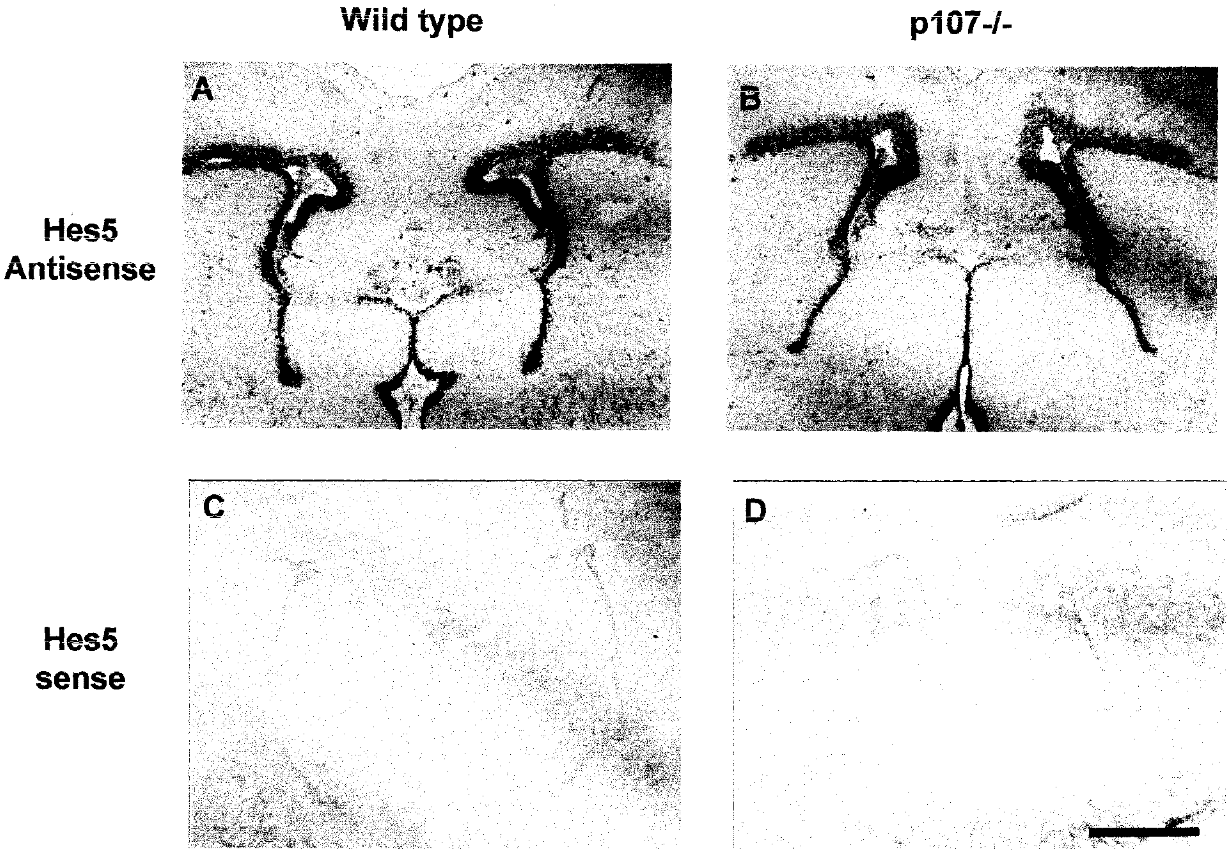
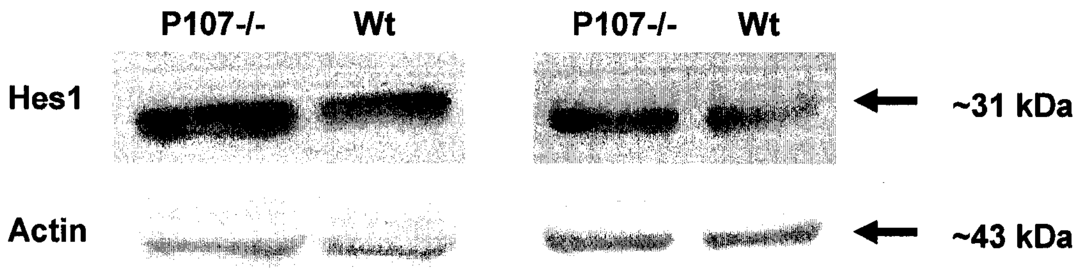

Wild type
p107-/-
Hes5
Antisense
A
B
Hes5
sense
C
D

Figure 8. **Hesl protein is increased in p107 -/- progenitor cells.** Neurospheres were cultured from p107-/- neuroepithelia at E10.5 and their wildtype littermates. Protein was isolated for western blot analysis and blots were probed with a mouse Hes1 primary antibody and anti-actin control. Hes1 protein is found at ~31 kDa and actin is found at ~ 43 kDa. (n = 3)


P107-/-
Wt
P107-/-
Wt
Hes1
Actin
~31 kDa
~43 kDa

***P107 and E2F4 bind to the same E2F binding site on the Notch1 regulatory sequences.*** The pocket protein family, of which p107 is a member, interacts with the E2F family of transcription factors (Ginsberg et al., 1994). P107, in particular, interacts preferentially with E2F4, forming a complex which actively represses transcription (Cobrinik, 2005). The E2F family recognizes E2F consensus sites found in the promoters of E2F responsive genes (Frolov

et al., 2004). Our laboratory previously showed that the Notch1 gene contained several E2F binding sites (Figure 9) and that p107 binds to an E2F binding site located in intron 1 (Vanderluit, 2004) (Figure 10A). We next questioned whether E2F4, a member of the E2F family that interacts with p107 to actively repress transcription, also bound to the same site in intron 1. To answer this question chromatin immunoprecipitation was performed on neurospheres cultured from wildtype E13.5 mice. Chromatin immunoprecipitation is a powerful technique allowing one to identify which transcription factors bind to promoter elements of a given gene in the context of native chromatin. Briefly, formaldehyde was added to crosslink all proteins interacting with DNA, after which the DNA was sonicated and E2F4 antibodies were added to pull-down E2F4 complexes bound to DNA. The crosslinks were reversed and the remaining DNA was analyzed by PCR using primers to amplify the first intron of the Notch1 gene. Our results show that E2F4 directly binds to the E2F site found on intron 1 of the Notch1 gene (Figure 10B). This is the same regulatory sequence that was previously shown to bind p107 (Vanderluit et al., 2004). Since p107 and E2F4 both bind the same regulatory sequence on the Notch gene, these results suggest that p107 regulates Notch1 gene expression through interactions with its target, E2F4. We next investigated whether p107 also interacted directly with the Hes1

Figure 9. **E2F binding sites on Notch1 promoter.** The Notch1 gene sequence previously characterized by del Amo et al. (1993), was examined using Genomatix promoter analysis software MatInspector. Putative E2F binding sites located throughout the gene are indicated by (*).

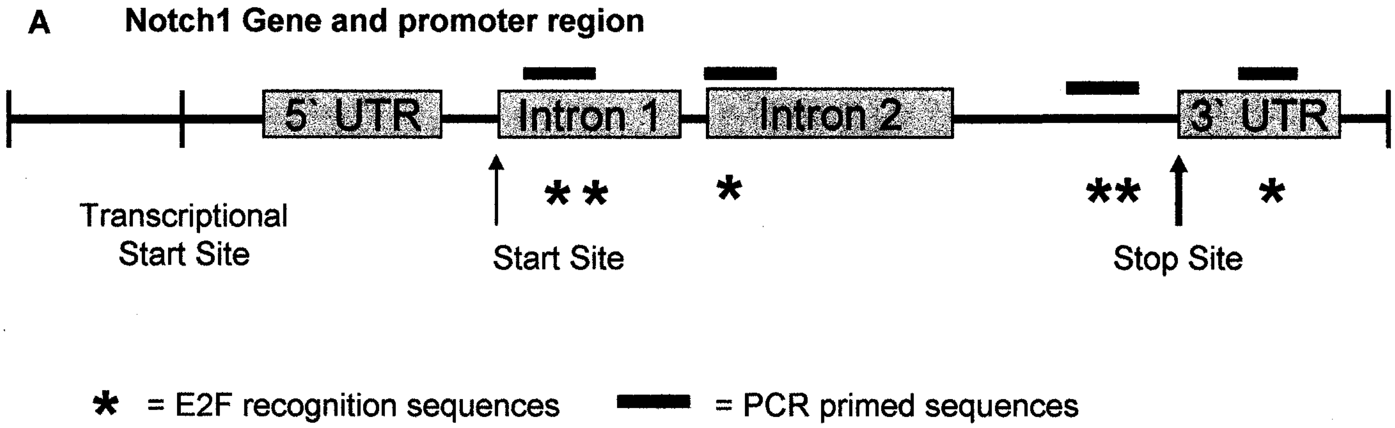
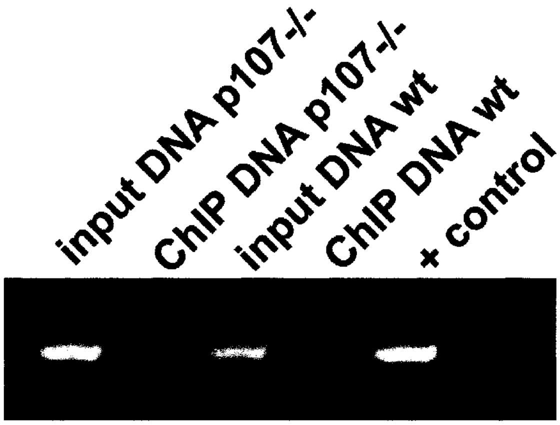
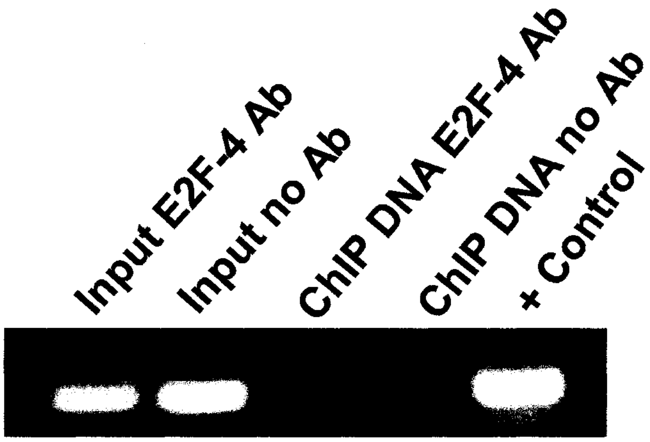

A
Notch1 Gene and promoter region
5` UTR
Intron 1
Intron 2
3` UTR
Transcriptional Start Site
Start Site
Stop Site
* = E2F recognition sequences
= PCR primed sequences

Figure 10. **p107 and E2F4 bind to the same E2F binding site on the Notch1 gene.** (A) Chromatin immunoprecipitation was performed on p107-/- and wildtype neurospheres. p107 antibody was used to immunoprecipitate all DNA bound to protein complexes containing p107. PCR was used with Notch1 primers that amplified an E2F binding site in intron 1. For each sample an aliquot of DNA was removed after sonication to ensure that the DNA had not been cut into fragments smaller than 200 bp. (B) Chromatin immunoprecipitation was performed on wildtype neurospheres using an E2F4 antibody to pull down all DNA bound to E2F4. PCR was used with Notch1 primers to amplify the E2F Binding site in intron 1.

A


input DNA p107-/-
ChIP DNA p107-/-
input DNA wt
ChIP DNA wt
+ control

B


Input E2F-4 Ab
Input no Ab
ChIP DNA E2F-4 Ab
ChIP DNA no Ab
+ Control

promoter by determining if the Hes1 promoter also contained E2F consensus sequences.

### *The Hes1 promoter contains three putative E2F binding sites.*

The selective elevation of Hes1 expression and not Hes5 lead us to question whether p107 directly regulated Hes1 gene transcription. The murine Hes1 promoter was previously characterized by Takebayashi et al. (1994). This promoter sequence and a 504 bp sequence following the Hes1 transcription start site was examined using the Genomatix promoter analysis software MatInspector. MatInspector locates putative transcription factor binding sites by comparing the promoter sequence to a database of experimentally verified transcription factor matrices (Quandt et al., 1995). Three putative E2F binding sites (E2F BS) were found on the Hes1 promoter at positions, +161 (E2F BS1), +398 (E2F BS2), and -560 (E2F BS3) relative to the transcription start site (Figure 11). E2F BS1 is located between the transcriptional start site and the translational start site. E2F BS2 is located in the middle of intron 1 and E2F BS3 is located in the middle of the identified promoter region. Table 2 shows the core and matrix values for each of the three sites. The core is determined by MatInspector to be the four consecutive nucleotides that have the highest consensus values among verified sites and the matrix refers to the entire binding sequence. The closer the core and matrix values are to 1.000, the higher the probability that the site is real. Core and matrix values are determined by an algorithm that computes how each individual base pair in the query sequence matrix matches up with the corresponding base pair in the predetermined "perfect" E2F binding matrix (Quandt et al., 1995). The presence of E2F binding sites on the Hes1 promoter suggests that Hes1 transcription may be regulated by one of the pocket proteins, possibly p107, through E2F. We next questioned

Figure 11. **The Hes1 promoter contains three putative E2F binding sites**. The Hes1 promoter sequence as previously determined by Takebashi et al. (1994) as well as a 504 bp sequence following the transcriptional start site was examined using the Genomatix promoter analysis software MatInspector. Three putative E2F binding sites were found on the Hes1 promoter at positions +161 (E2F BS1), +398 (E2F BS2), and -560 (E2F BS3) relative to the transcriptional start site. E2F BS1 is located between the transcriptional start site and the translational start site. E2F BS2 is located in the middle of intron 1. E2F BS3 is located in the middle of the identified promoter region. CAAT and TATA boxes are shown by black boxes ■. N Boxes are shown with lined boxes ▨, and E2F sites are shown by empty boxes □.

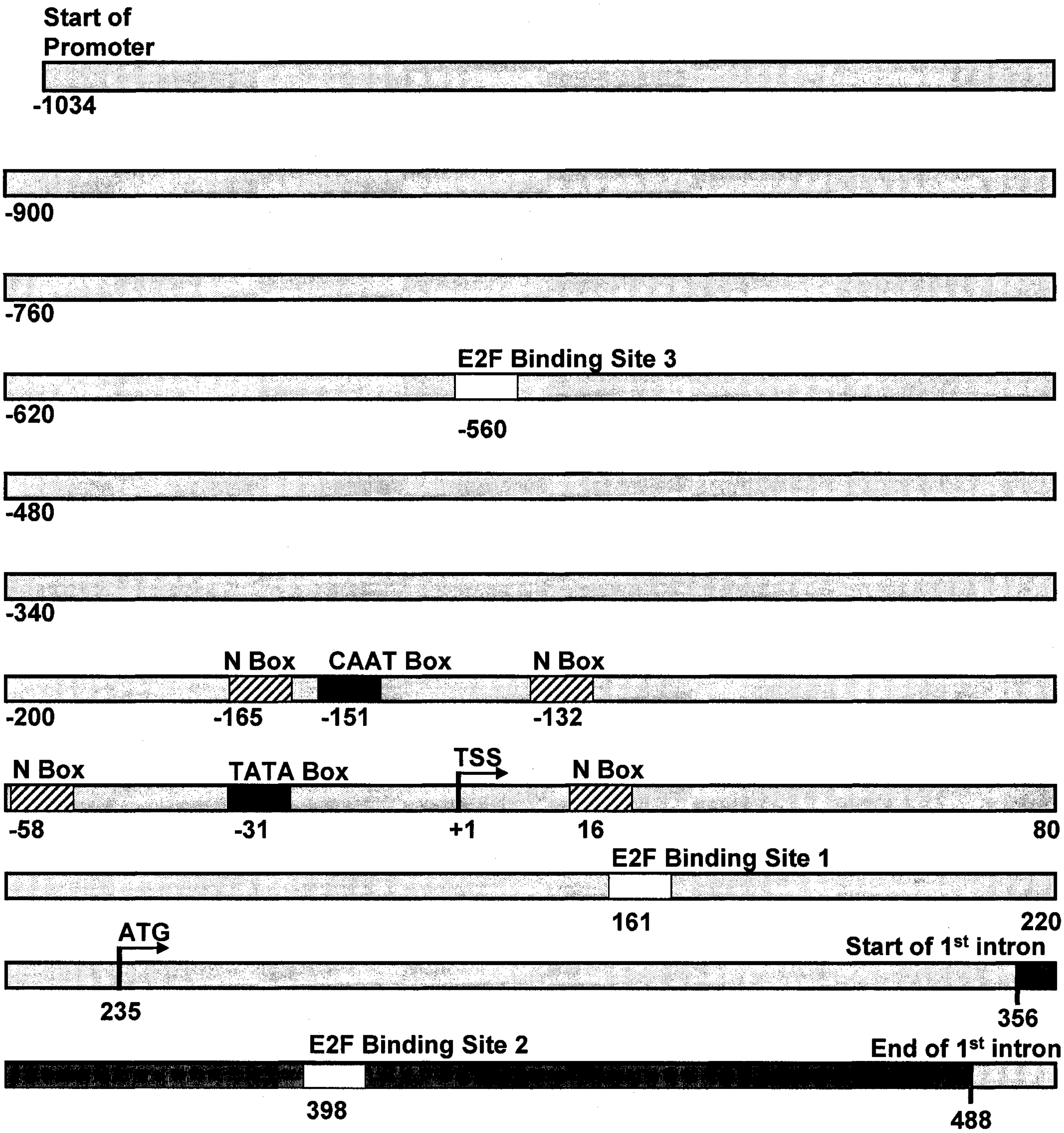

Start of Promoter
-1034
-900
-760
E2F Binding Site 3
-620
-560
-480
-340
N Box
CAAT Box
N Box
-200
-165
-151
-132
N Box
TATA Box
TSS
N Box
-58
-31
+1
16
80
E2F Binding Site 1
161
220
ATG
Start of 1st intron
235
356
E2F Binding Site 2
End of 1st intron
398
488

Table 2: Three putative E2F binding sites on Hes1 promoter identified by MatInspector

| **Site Name** | **Position from Transcriptional Start site** | **Core Value** | **Matrix Value** | **Sequence** |
|---|---|---|---|---|
| E2F BS1 | +161 | 0.857 | 0.843 | ttcacgcAAAAaaaa |
| E2F BS2 | +398 | 1.000 | 0.744 | tttaagaGAAAagta |
| E2F BS3 | -560 | 0.857 | 0.843 | cacacgcTAAAcatg |

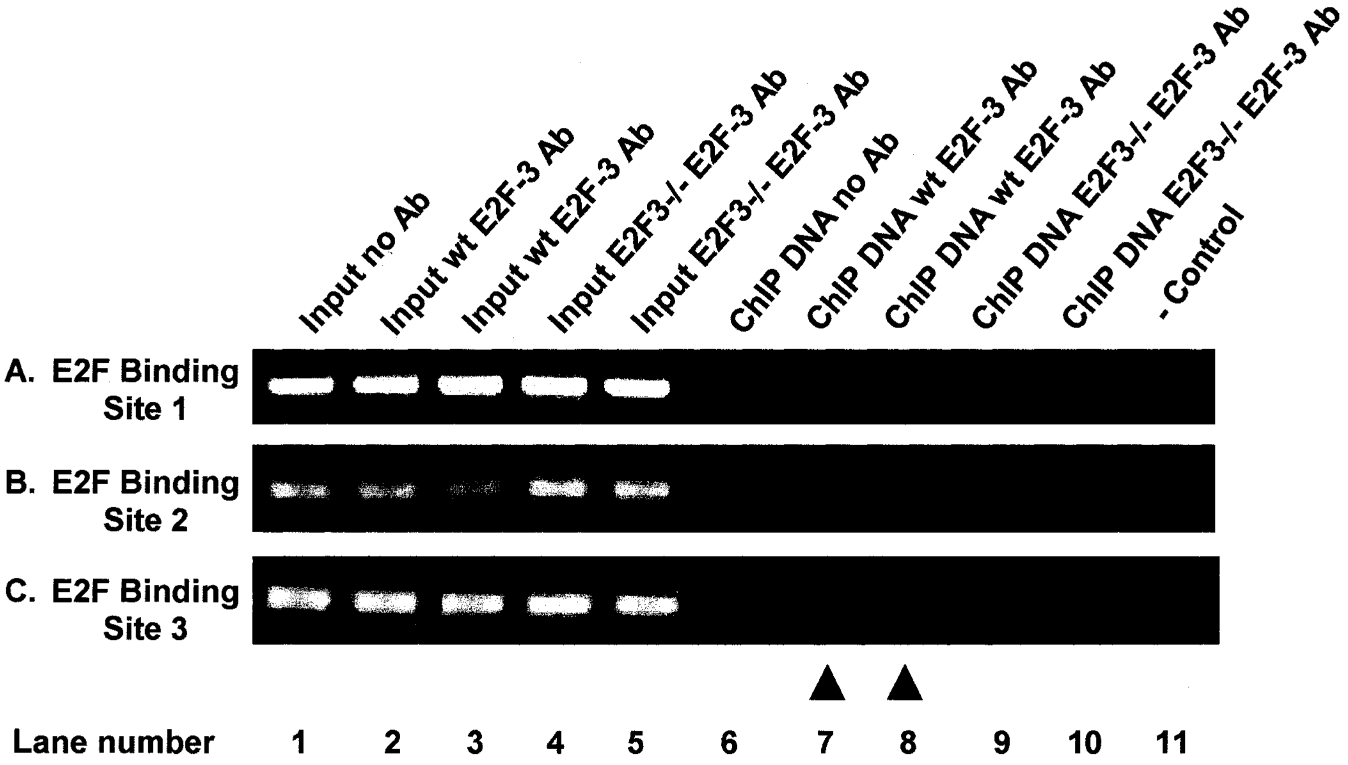
Note: the core of the binding site is indicated by capital letters and matrix is indicated by lowercase letters.

whether these sites were active, whether there was E2F binding activity, and which E2Fs were interacting at those sites.

### ***Both E2F3 and E2F4 bind to the E2F binding sites on the Hes1 promoter.***

To determine if the three E2F sites on the Hes1 promoter are occupied by E2Fs in neural precursors we used chromatin immunoprecipitation. Since our data suggested that Hes1 transcription is repressed by p107, we examined those E2Fs that are known transcriptional repressors such as E2F3b and E2F4 (Attwooll et al., 2004). Neurospheres were cultured from E14.5 wildtype and E2F3-/- embryos. The DNA was immunoprecipitated with an E2F3 antibody that detects both E2F3a and E2F3b, and was amplified with primers designed around the three putative E2F binding sites on the Hes1 promoter (Table 1). The following three controls were used in this experiment. An aliquot of DNA, designated input DNA, was removed from each sample after sonication to ensure that the DNA had not been cut into fragments smaller than 200 bp which would have resulted in the primers no longer being able to amplify the appropriate regions and giving a false negative. Furthermore, E2F3-/- neurospheres were used to ensure that the E2F3 antibody was not cross-reacting with other members of the E2F family resulting in a false positive (lanes 9 and 10, Figure 12). Finally, a negative control was used in the PCR reaction, where no DNA was added, to ensure that none of the PCR reagents were contaminated with DNA. There were bands in all of the input DNA lanes, indicating that the DNA was intact (lanes 1 through 5, Figure 12) and there were no bands in the negative control or the E2F3-/- lanes indicating that there was no contamination in the PCR reaction and that the E2F3 antibody did not cross-react. E2F3 binding activity was found at E2F

Figure 12. **E2F3 binds to E2F binding sites 1 and 3 on the Hes1 promoter.** Chromatin immunoprecipitation was performed on E 14.5 wildtype and E2F3-/- neurospheres. The DNA was immunoprecipitated with an E2F3 antibody that detects both E2F3a and E2F3b, and was amplified with primers designed around the three putative E2F binding sites on the Hes1 promoter (Table 1). The following three controls were used in this experiment. An aliquot of DNA, designated input DNA, was removed from each sample after sonication to ensure that the DNA had not been cut into fragments smaller than 200 bp which would have resulted in the primers no longer being able to amplify the appropriate regions and giving a false negative. Furthermore, E2F3-/- neurospheres were used to ensure that the E2F3 antibody was not cross-reacting with other members of the E2F family resulting in a false positive (lanes 9 and 10). Finally, a negative control was used in the PCR reaction, where no DNA was added, to ensure that none of the PCR reagents were contaminated with DNA. There were bands in all of the input DNA lanes, indicating that the DNA was intact (lanes 1 through 5) and there were no bands in the negative control or the E2F3-/- lanes indicating that there was no contamination in the PCR reaction and that the E2F3 antibody did not cross-react. E2F3 binding activity was found at E2F Binding sites 1 and 3 (lanes 7 and 8, Figure 9A,C) but not at E2F binding site 2 (lanes 7 and 8, B). (n = 3)

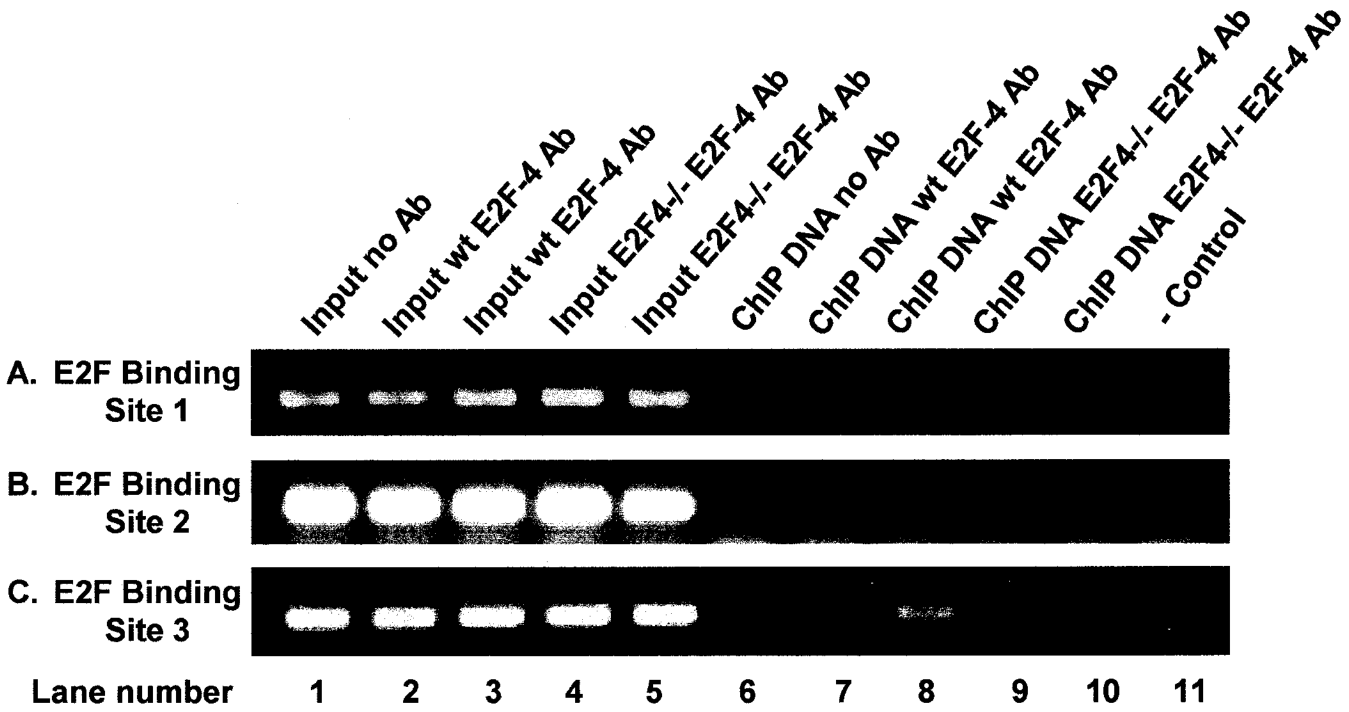

Input no Ab
Input wt E2F-3 Ab
Input wt E2F-3 Ab
Input E2F3-/- E2F-3 Ab
Input E2F3-/- E2F-3 Ab
ChIP DNA no Ab
ChIP DNA wt E2F-3 Ab
ChIP DNA wt E2F-3 Ab
ChIP DNA E2F3-/- E2F-3 Ab
ChIP DNA E2F3-/- E2F-3 Ab
- Control
A. E2F Binding Site 1
B. E2F Binding Site 2
C. E2F Binding Site 3
Lane number
1
2
3
4
5
6
7
8
9
10
11

Binding sites 1 and 3 (lanes 7 and 8, Figure 12A,C) but not at E2F binding site 2 (lanes 7 and 8, Figure 12B).

Since E2F4 is the preferential binding partner for p107, we examined whether E2F4 was capable of binding to any of the three E2F sites using chromatin immunoprecipitation. Neurospheres were cultured from E13.5 E2F4-/- and wildtype embryos. DNA was immunoprecipitated with E2F4 antibodies and amplified with primers designed around the putative E2F binding sites (Table 1). Again the same three controls were used as mentioned above. The input DNA revealed that the all DNA was intact, and the absence of bands in the negative and E2F4-/- control lanes indicated that there was no contamination in the PCR reaction and that the E2F4 antibody was not cross-reacting (lanes 9 and 10, Figure 13). We found E2F4 binding activity at E2F binding site 3 on the Hes1 promoter (Figure 13 C). There was no observed binding activity at binding site 1 and very weak binding activity at binding site 2 (Figure 13 B).

These results suggest that the putative E2F binding sites on the Hes1 promoter exhibit E2F binding activity in the native chromatin of wildtype neural precusor cells. There was binding primarily at E2F sites 1 and 3 and to a lesser extent site 2. E2F3 binds to sites 1 and 3, and E2F4 preferentially binds to sites 3 and 2. We next questioned whether these sites were capable of actively repressing or inducing Hes1 promoter activity in response to p107, E2F3, and E2F4 using a standard Luciferase reporter assay.

Figure 13. **E2F4 binds to E2F binding site 3 on the Hes1 promoter.** Chromatin immunoprecipitation was performed on E 13.5 wildtype and E2F4-/- neurospheres. DNA was immunoprecipitated with E2F4 antibodies and amplified with primers designed around the putative E2F binding sites (Table 1). The following three controls were used in this experiment. An aliquot of DNA, designated input DNA, was removed from each sample after sonication to ensure that the DNA had not been cut into fragments smaller than 200 bp which would have resulted in the primers no longer being able to amplify the appropriate regions and giving a false negative. Furthermore, E2F4-/- neurospheres were used ensure that the E2F4 antibody was not cross-reacting with other members of the E2F family resulting in a false positive (lanes 9 and 10). Finally, a negative control was used in the PCR reaction, where no DNA was added, to ensure that none of the PCR reagents were contaminated with DNA. The input DNA revealed that the all DNA was intact, and the absence of bands in the negative and E2F4-/- control lanes indicated that there was no contamination in the PCR reaction and that the E2F4 antibody was not cross-reacting (lanes 9 and 10). We found E2F4 binding activity at E2F binding site 3 on the Hes1 promoter. There was no observed binding activity at binding site 1 and very weak binding activity at binding site 2 (B). (n = 3)

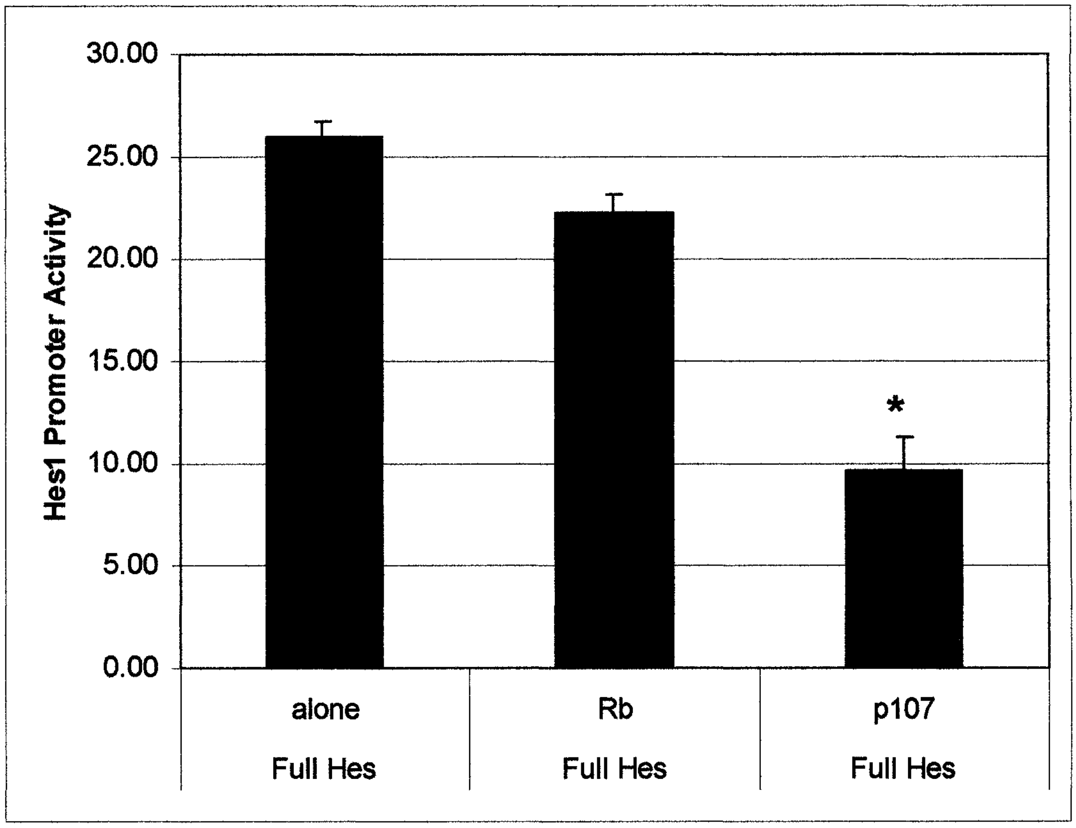

Input no Ab
Input wt E2F-4 Ab
Input wt E2F-4 Ab
Input E2F4-/- E2F-4 Ab
Input E2F4-/- E2F-4 Ab
ChIP DNA no Ab
ChIP DNA wt E2F-4 Ab
ChIP DNA wt E2F-4 Ab
ChIP DNA E2F4-/- E2F-4 Ab
ChIP DNA E2F4-/- E2F-4 Ab
- Control
A. E2F Binding Site 1
B. E2F Binding Site 2
C. E2F Binding Site 3
Lane number
1
2
3
4
5
6
7
8
9
10
11

### *P107 represses Hes1 promoter activity.*

The chromatin immunoprecipitations with E2F3 and E2F4 antibodies indicated that there was E2F binding activity at the E2F sites on the Hes1 promoter. P107, and other pocket proteins including pRb, regulate transcription through the E2Fs (Cobrinik, 2005). We used the Luciferase Reporter assay to determine if the Hes1 promoter was responsive to p107 or pRb. This assay measures whether a promoter is responsive to a transcription factor or its regulatory proteins and indicates whether they have a repressive or activating effect. The Hes1 promoter (~1500 bp), including all three putative E2F binding sites, was inserted into the pGL3-Basic vector (Promega) containing the luciferase gene. This construct was transfected into HEK 293 cells using calcium phosphate precipitation according to the protocol of Charest et al. (1993), along with 3 μg of expression vectors containing either Rb (pGK-RB) or p107 (pCMV-p107) and 2 μg of pGK-LacZ as a control. MUGs assay was performed to standardize the transfection reaction by determining the rate at which B-galactosidase converts 4-methylumbelliferyl-D-galactoside (MUG) (Sigma-Aldrich) to methylumbelliferone, a fluorescent molecule. The ratio of β-galactosidase activity to luciferase activity was used to normalize all of the treatments. Rb had no effect on Hes1 promoter activity, however p107 significantly ($P < 0.05$) repressed the activity of the Hes1 promoter by a factor of 2.5 (Figure 14). This result is consistent with our previous findings that Hes1 transcript and protein are upregulated in p107 deficient brains and demonstrates that this repression is specific to p107 and not pRB.

Having found that E2F3 and E2F4 interact with the Hes1 promoter, we questioned whether p107 could repress promoter activity through E2F3 or E2F4. The Hes1 promoter construct was transfected into HEK 293 cells alone and in combination with expression

Figure 14. **p107 represses Hes1 promoter activity.** Luciferase assay of HEK 293 cells transfected using calcium phosphate precipitation method with Hes1 promoter constructs and 3ug of expression vectors containing either Rb (pGK-RB) or p107 (pCMV-p107). Rb did not effect Hes1 promoter activity, however p107 significantly represses the activity of the Hes1 promoter by a factor of 2.5 ($P < 0.05$) ($n = 3$).


Hes1 Promoter Activity
30.00
25.00
20.00
15.00
10.00
5.00
0.00
*
alone
Full Hes
Rb
Full Hes
p107
Full Hes

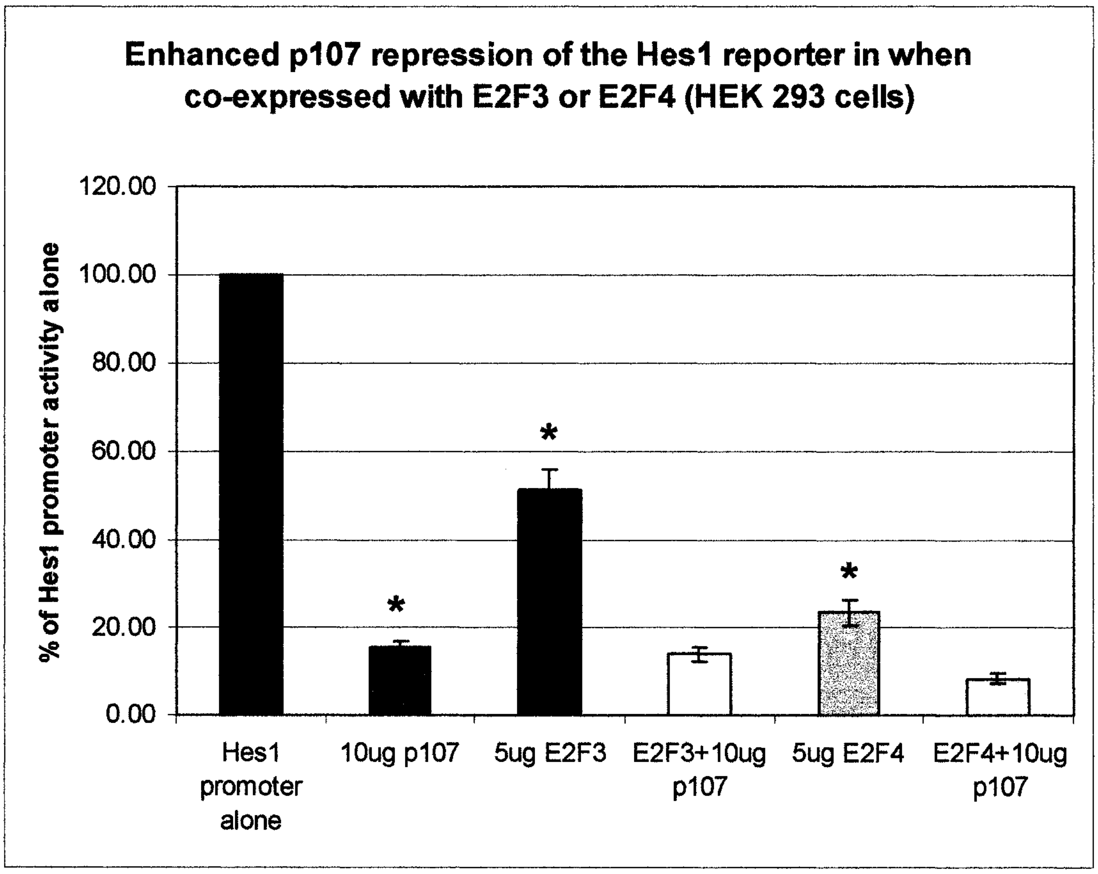
vectors for either E2F3 (pCMV-E2F3), E2F4 (pCMV-E2F4), and p107 (pCMV-p107). 5 μg of pCMV-E2F3 and pCMV-E2F4 expression vectors and 10 μg of pCMV-p107 were used along with 2 μg of pGK-LacZ control. The amount of p107 (pCMV-p107) DNA transfected was increased from 3 μg to 10 μg from the previous experiment due to concerns about the stability of p107 protein expression. A significant two-fold repression was observed of the Hes1 promoter by E2F3 alone and significant four fold repression by E2F4 alone ($P < 0.05$) (Figure 15 A). Repression of the Hes1 promoter was enhanced when P107 and E2F4 were coexpressed relative to p107 expression alone (Figure 15 B). These results demonstrate that the Hes1 promoter is responsive to p107 and its regulatory targets E2F3 and E2F4. We next asked if Hes1 was deregulated in either E2F3 or E2F4 deficient neural precusors.

### *Hes1 protein levels are not upregulated in E2F3-/- or E2F4-/- neurospheres.*

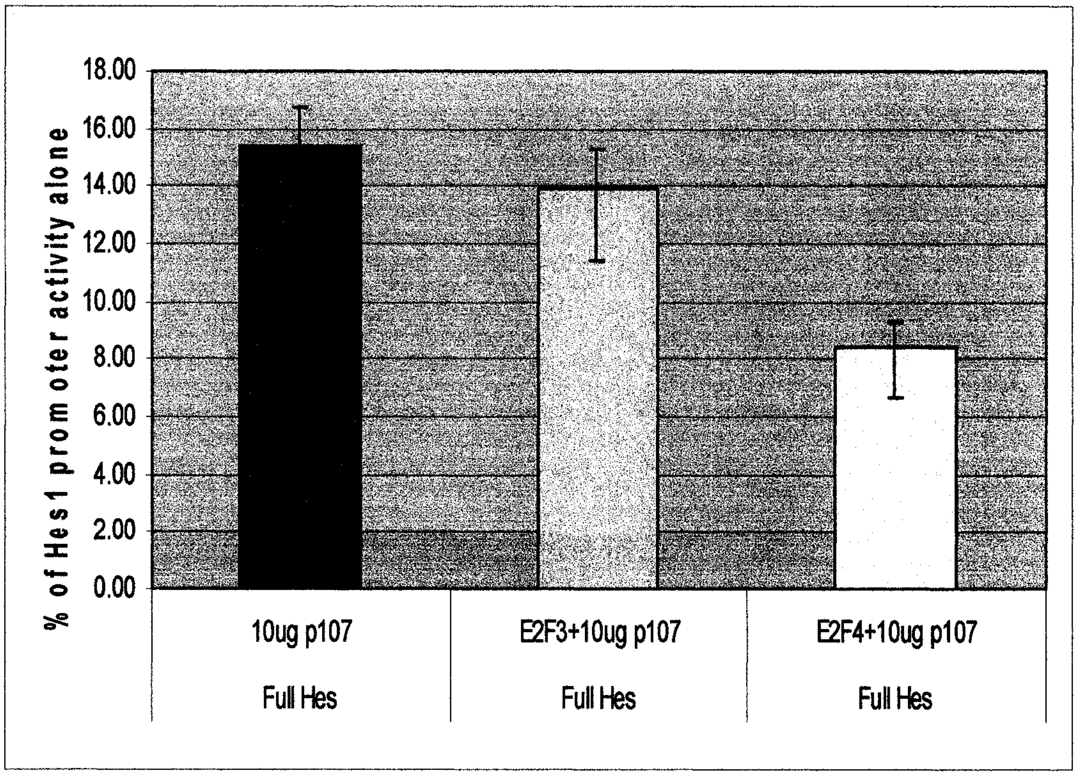
The Luciferase reporter assay showed that Hes1 promoter activity could be repressed by E2F3 and E2F4. We questioned whether E2F3 or E2F4 expression was required for p107-mediated repression of Hes1. To address this question we examined the overall levels of Hes1 expression in wildtype and E2F3-/- and E2F4-/- neural precursor cells. If p107 requires either E2F3 or E2F4 expression in order to repress Hes1, we would expect elevated levels of Hes1 in either the E2F3-/- or E2F4-/- neurospheres. The ganglionic eminence was dissected from E2F3-/- E14.5 embryos and their wildtype littermates, dissociated into single cells and cultured to form neurospheres. Protein was extracted and western blot analysis was performed using Hes1 antibody (Ito et al., 2000). No difference

Figure 15. **Coexpression of E2F4 and p107 further represses Hes1 promoter activity.** The full Hes1 promoter construct was transfected into HEK 293 cells alone and in combination with expression vectors for either E2F3 (pCMV-E2F3), E2F4 (pCMV-E2F4), and p107 (pCMV-p107). 5 µg of pCMV-E2F3 and pCMV-E2F4 expression vectors were used and 10 µg of CMV-p107. Hes1 promoter activity was significantly repressed in the presence of E2F3 or E2F4 alone by a factor of 2 and 4 respectively ($P < 0.05$). Hes1 promoter activity was further repressed with the coexpression of p107 with E2F4. (B) Same information as in A only showing trials with pCMV-p107, pCMV-E2F3 + pCMV-p107, and pCMV-E2F4 + pCMV-p107. ($n = 3$)

A


Enhanced p107 repression of the Hes1 reporter in when co-expressed with E2F3 or E2F4 (HEK 293 cells)
% of Hes1 promoter activity alone
120.00
100.00
80.00
60.00
40.00
20.00
0.00
*
*
*
Hes1 promoter alone
10ug p107
5ug E2F3
E2F3+10ug p107
5ug E2F4
E2F4+10ug p107

B


% of Hes1 promoter activity alone
18.00
16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
10ug p107
Full Hes
E2F3+10ug p107
Full Hes
E2F4+10ug p107
Full Hes

was found between the levels of Hes1 protein in E2F3-/- neurospheres relative to their wildtype littermates (Figure 16).

Western blot analysis was also used to determine if there was an increase of Hes1 protein in E2F4 -/- neural precursor cells. Neurospheres were cultured from E2F4-/- E14.5 embryos and their wildtype littermates. Protein was extracted from neurospheres and western blots were incubated with Hes1 antibody (Ito et al., 2000). No difference was found in the protein levels of Hes1 in E2F4-/- neural precursor cells relative to wildtype (Figure 17).

These results demonstrate that Hes1 protein is not deregulated in either E2F3-/- or E2F4-/- neural precursor cells. Furthermore, removing either E2F3 or E2F4 does not impair the ability of p107 to repress Hes1 expression. P107 is still present in both of these E2F deficient conditions and may be recruiting other E2Fs to the Hes1 promoter, thereby maintaining proper levels of Hes1 expression. Since both E2F3 and E2F4 are capable of repressing Hes1 expression, deletion of both E2F3 and E2F4 may be required to alleviate p107 mediated repression of Hes1 expression.

### ***P107 regulates neural precursor cell number through Hes1.***

Our group previously found an increase in the number of neural precursor cells and in their self-renewal ability in P107-/- embryos (Vanderluit et al, 2004). Studies have shown that the Notch pathway is required for neural stem cell self-renewal (Hitoshi et al., 2002b; Nakamura et al., 2000). Taken together with the results demonstrating that the Notch-Hes1 signalling pathway is upregulated in p107-/- embryos and that p107 represses Hes1 expression, we next asked if the increased neural precursor cell number observed in p107-/-

Figure 16. **Hes1 protein levels are not upregulated in E2F3-/- neurospheres.** The ganglionic eminence was dissected from E2F3-/- E14.5 embryos and their wildtype littermates, dissociated into single cells and cultured to form neurospheres. Protein was extracted and western blot analysis was performed using Hes1 antibody (Ito et al., 2000). No difference was detected between the levels of Hes1 protein in E2F3 -/- neurospheres compared to their wildtype littermates. (n = 2)

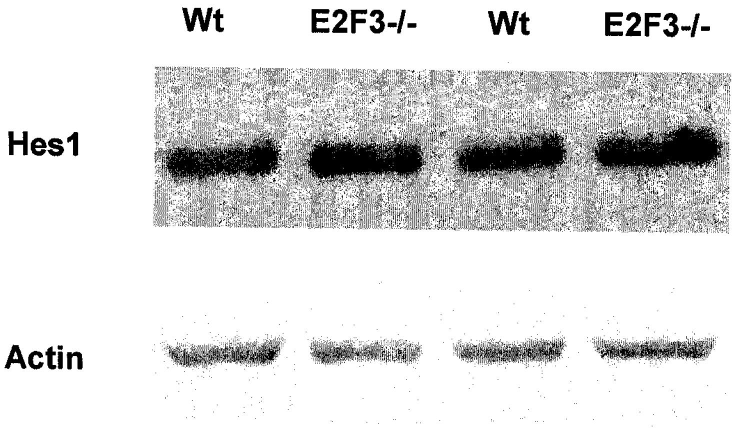
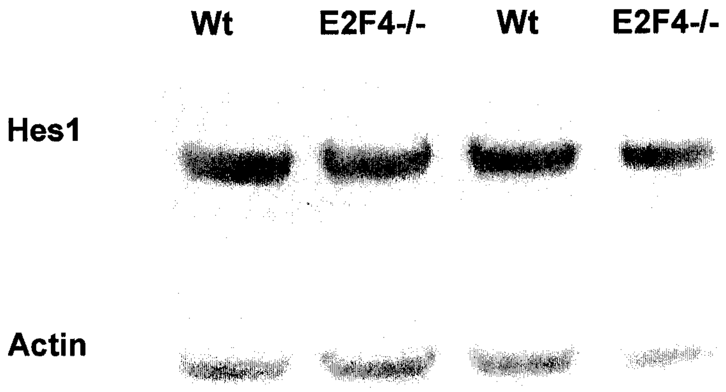

Wt
E2F3-/-
Wt
E2F3-/-
Hes1
Actin

Figure 17. **Hes1 protein levels are not upregulated in E2F4-/- neurospheres.** The ganglionic eminence was dissected from E2F4-/- E13.5 embryos and their wildtype littermates and neurospheres were cultured. Protein was extracted and western blot analysis was performed using Hes1 antibody (Ito et al., 2000). Similar levels of Hes1 protein were detected in E2F4-/- and wildtype neurospheres. (n = 2)

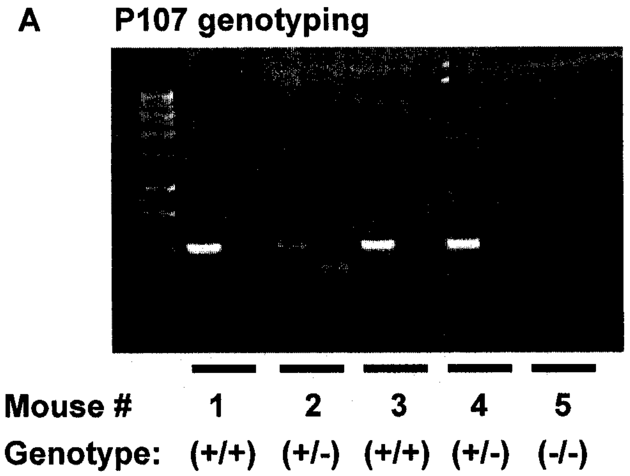

Wt
E2F4-/-
Wt
E2F4-/-
Hes1
Actin

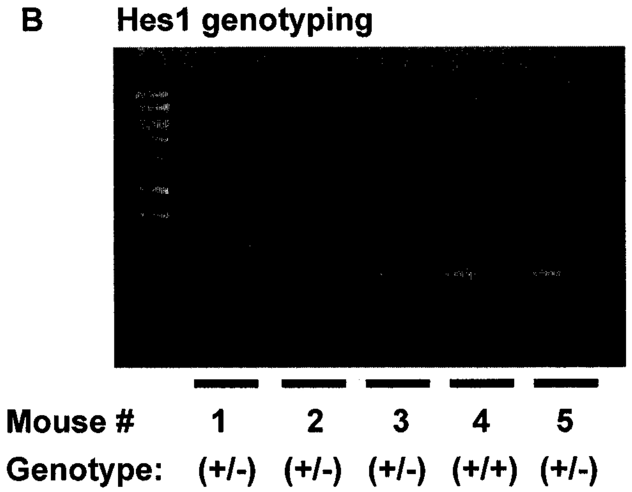
embryos was due to an upregulation of the Notch-Hes1 signalling pathway. To address this question, p107 deficient mice were crossed with Hes1 deficient mice and neural precursor cell number was examined using the primary neurosphere assay. The primary neurosphere assay is an in vitro technique used to quantify the neural stem cell population, on the basis that each neural precursor will give rise to a neurosphere in culture (Reynolds et al, 1992a). If the increased number of neural precursor cells in p107-/- mice is due to enhanced levels of Notch1 and Hes1, then deletion of a downstream member of that pathway, such as Hes1, should restore the number of neurosphere forming cells to wildtype levels in p107-/- embryos.

P107 -/- mice, which were previously characterized by LeCouter et al. (1998b), are slightly runted as pups but are indistinguishable from their wildtype littermates when full grown. Ishibashi et al (1995) previously showed that Hes1-/- mice on an ICR background are embryonic lethal at E14.5 due to neural tube closure defects and premature neuronal differentiation. Hes1 heterozygous mice however, are viable and fertile and show no apparent phenotype. To generate mice deficient in both p107 and Hes1, double heterozygous (Hes +/-; p107 +/-) mice were bred.

Figure 18 is a typical ethidium bromide agarose gel showing PCR amplification of both the wildtype and mutant genes of p107 and Hes1, which was used to genotype these mice according to the protocols previously described by LeCouter et al. (1998b) and Ishibashi et al. (1995) respectively. The fragment size for the wildtype p107 allele amplification product is ~184 bp and ~120 bp for the mutant allele. The fragment size for the Hes1 wildtype allele amplification product is 122 bp and the Hes1 mutant allele is 476 bp. When the PCR amplification products are electrophoresed through an agarose gel,

Figure 18. **P107 and Hes1 Genotyping.** Ethidium bromide agarose gel showing PCR amplification of both the wildtype and mutant genes for p107 (A) and Hes1(B), which is used to genotype these mice for further experimentation. The fragment size for p107 amplification product is ~184 bp for the wildtype allel and ~120 bp for the mutant allel. The fragment size for Hes1 wildtype is 122bp and for Hes1 mutant is 476. The genotypes of the mice in this example gel are 1: (p107 +/+; Hes1 +/-), 2: (p107 +/-; Hes1 +/-), 3: (p107 +/+; Hes1 +/-), 4: (p107 +/-; Hes1 +/+), and 5: (p107 -/-; Hes1 +/-).


A
P107 genotyping
Mouse #
1
2
3
4
5
Genotype:
(+/+)
(+/-)
(+/+)
(+/-)
(-/-)


B
Hes1 genotyping
Mouse #
1
2
3
4
5
Genotype:
(+/-)
(+/-)
(+/-)
(+/+)
(+/-)

mice that only have a band in the wildtype lane were genotyped homozygous for the wildtype allele. Mice that have bands in both the wildtype and mutant lanes were genotyped heterozygous and possess both a mutant and wildtype allele, and mice that only have a band in the mutant lane were genotyped as homozygous for the mutant allele. Since Hes1-/- embryos die at E14.5, we examined embryos at E10.5. Due to the mixing of genetic backgrounds, wildtype littermates were always used as controls. The telencephalic neuroepithelium was dissected, the tissue dissociated into single cells and plated. Neurospheres were counted seven days later. The following genotypes were examined p107-/-, Hes1+/-, Hes1-/-, Hes1+/-; p107+/-, Hes1 +/-; p107-/-, Hes1-/-; p107-/- and wildtype. Figure 19 depicts representative neurosphere cultures from the examined genotypes. The sizes of neurospheres from double null Hes1-/-; p107-/- cultures were on average smaller compared to wildtype. The neurosphere size for the rest of the examined genotypes was comparable to wildtype.

Neurospheres smaller than 50 μm were not counted. Consistent with previous findings we observed no difference in the number of neurospheres in either Hes1+/- or Hes1-/- embryos compared to wildtype (Ohtsuka et al, 2001). We observed a significant increase in the number of p107-/- neurospheres relative to wildtype which was consistent with our group's previous findings (Vanderluit et al., 2004). When the gene dosage of Hes1 was halved in p107-/- embryos (Hes1+/-; p107-/-), the number of neurospheres was significantly reduced compared to p107-/- embryos containing only wildtype Hes1. When Hes1 was completely removed from p107-/- embryos (Hes1-/-; p107-/-), there was no significant difference in neurosphere number compared to wildtype controls (Figure 20).

Figure 19. **Primary neurospheres cultured from embryos arising from a double heterozygous (Hes1+/-; p107+/-) breeding.** Embryos were dissected at E10.5, neuroepithelium was extracted and neural precursor cells were cultured in serum-free media to generate neurospheres. Representative pictures of neurosphere cultures from the following genotypes at 7 days in vitro: (A) Wildtype, (B) p107-/-, (C) Hes1-/-;p107-/-, (D) Hes+/-;p107-/-, (E) Hes1-/-, and (F) Hes1+/-; p107+/-. Scale bar = 500 μm.

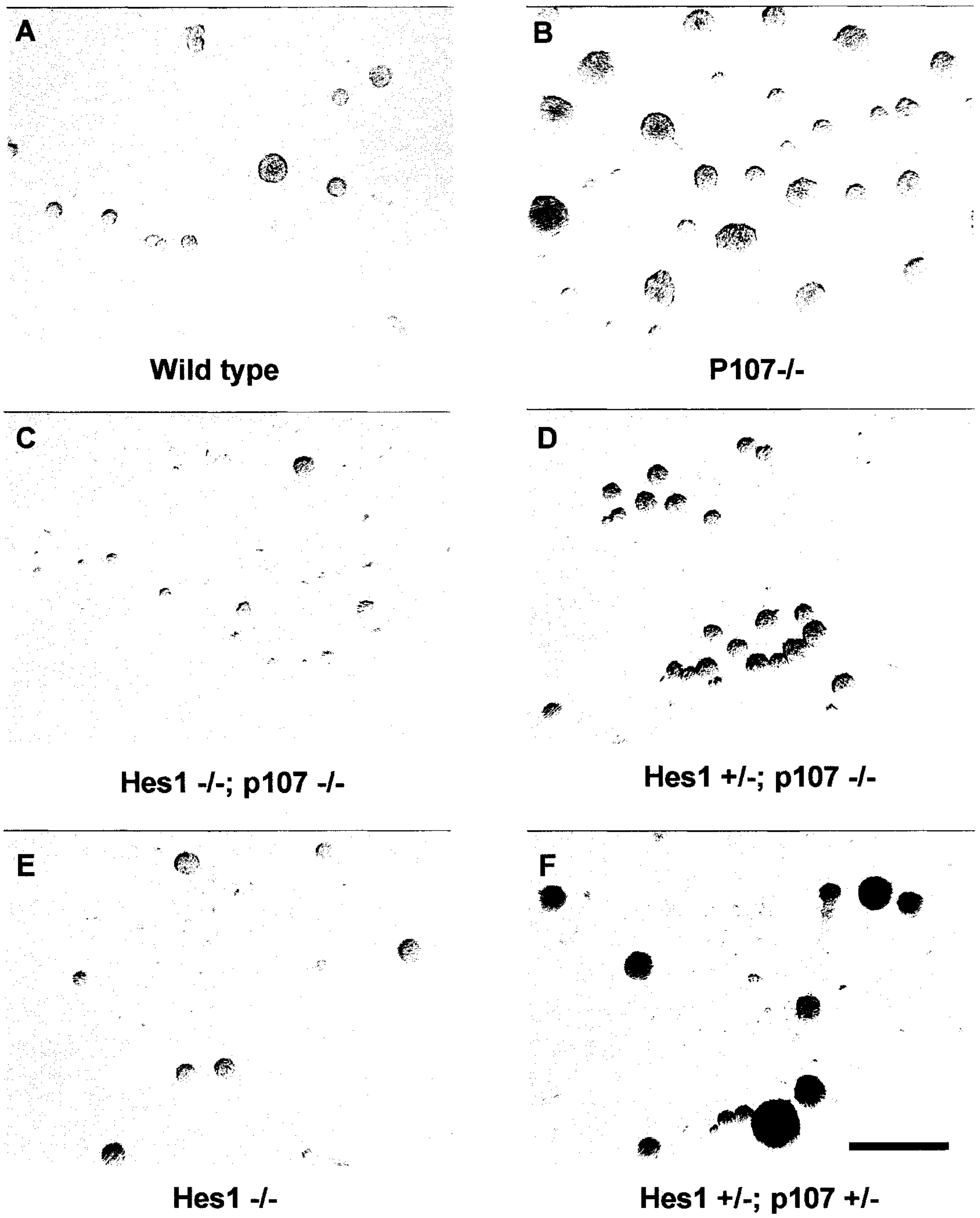
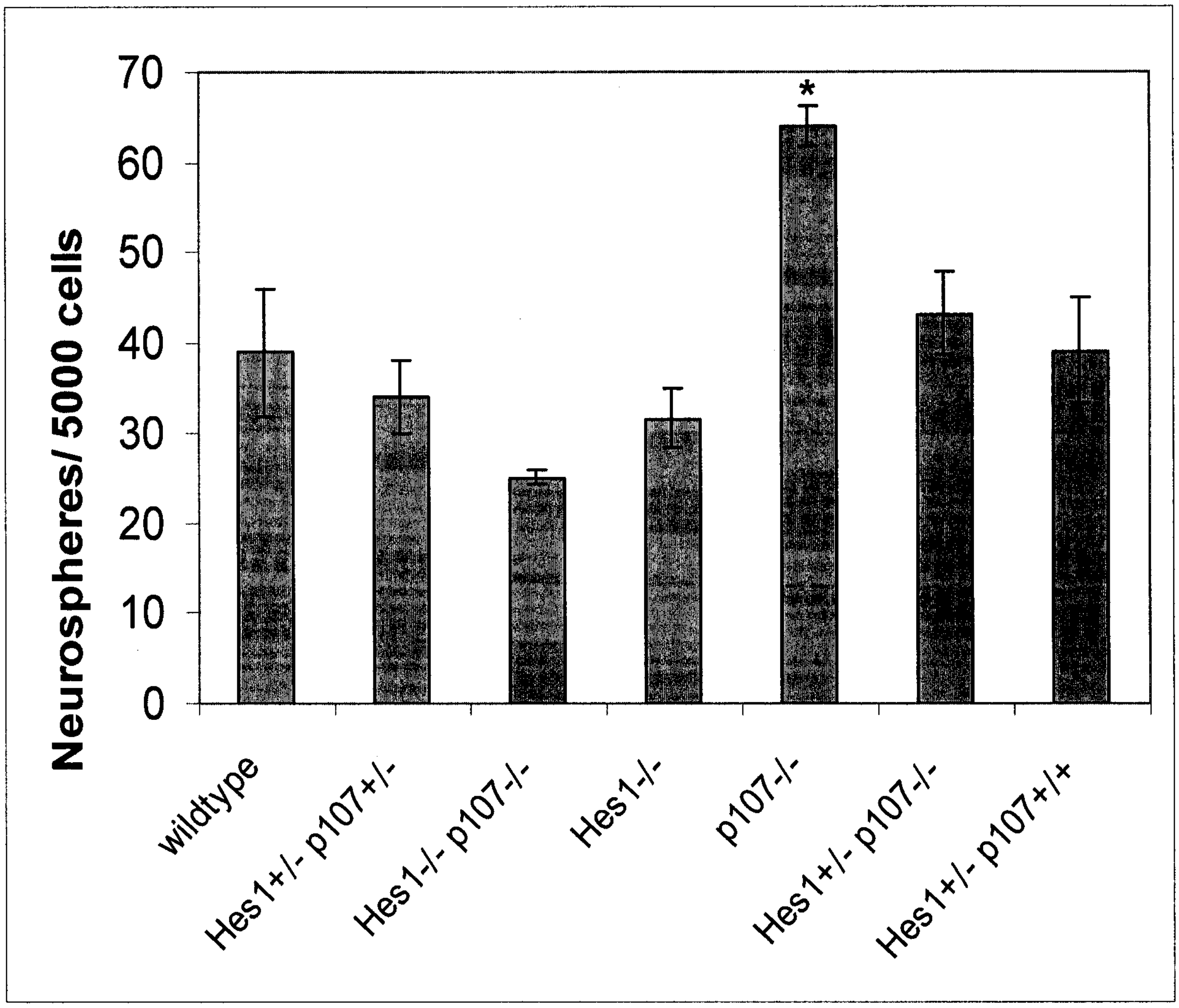

A
Wild type
B
P107-/-
C
Hes1 -/-; p107 -/-
D
Hes1 +/-; p107 -/-
E
Hes1 -/-
F
Hes1 +/-; p107 +/-

Figure 20. **P107 regulates neural precursor cell number through the Notch-Hes1 signalling pathway.** To assess neural stem cell numbers, the neurosphere assay from (Reynolds et al., 1992a) was used. Briefly, telencephalic neuroepthilium from embryonic day 10 embryos was dissected, neural precursor cells dissociated, and cultured in serum-free media to generate neurospheres. Cells were plated at 5000 cells/well and neurosphere counts were performed seven days later. At least 3 embryos were used of each genotype. Significant differences were assessed at $\alpha < 0.05$ and a Tukey's post hoc test was performed to detect differences in the means at $p < 0.05$.


Neurospheres/ 5000 cells
70
60
50
40
30
20
10
0
*
wildtype
Hes1+/- p107+/-
Hes1-/- p107-/-
Hes1-/-
p107-/-
Hes1+/- p107-/-
Hes1+/- p107+/+

These results demonstrate that the elevated number of neural precursor cells in p107-/- mice is due to enhanced expression of Hes1, since Hes1 gene deletion in p107-/- embryos restores the number of neural stem cells to wildtype levels. This finding supports our hypothesis that p107 is regulating the neural precursor cell population through the Notch-Hes1 signalling pathway.

## Discussion

The results of these studies yield several important findings. First, by in situ hybridization and western blot analysis we show that Hes1 transcript and protein levels are upregulated in p107-/- brains. Second, examination of the Hes1 promoter revealed three putative E2F binding sites. Third, chromatin immunoprecipitation experiments showed that E2F3 and E2F4 bound to the promoter. Fourth, p107 was found to significantly repress Hes1 promoter activity using the luciferase reporter assay. This repression was specific to p107 and not pRb. E2F3 and E2F4 were also found to significantly repress the Hes1 promoter. Finally we show that upregulation of Hes1 in p107-/- neural precursors is responsible for the increased neural precursor cell pool, since removal of Hes1 from p107-/- mice restores the increased level of neurosphere forming cells, normally seen in p107-/- mice (Vanderluit et al., 2004) to wildtype levels. In conclusion p107 regulates neural precursor cells by repression of the Hes1 promoter.

### *The Notch1-Hes1 signalling pathway is upregulated in p107-/- neural precursors.*

The levels of full length Notch1 and activated Notch (NICD) were previously found to be upregulated in p107 -/- neurospheres using western blot analysis (Vanderluit et al. (2004). Hes1 and Hes5 are both known Notch effectors (Ohtsuka et al., 1999). Consistent with increased Notch1 activity, we showed an upregluation of Hes1 transcript levels in p107-/- embryo brains using *in situ* hybridization as well as an upregulation in the protein levels of Hes1 in p107-/- neurospheres using western blot analysis. An increase in the Notch pathway is consistent with findings that p107-/- neural precursors have increased self-renewal, since the Notch pathway has also been shown to regulate stem cell self-renewal (Hitoshi et al., 2002b; Nakamura et al., 2000).

There was no difference in the transcript levels of Hes5, also a known Notch effector (Ohtsuka et al., 1999). This suggested to us that p107 was specifically upregulating Hes1 possibly through direct transcriptional regulation. Other groups have found instances where Notch1 overexpression upregulates only Hes1 or Hes5 but not both. For example Wu et al. (2003) found that when the active form of Notch (NICD) was overexpressed using a virus in neural progenitor cells isolated from rat spinal cord, there was an increase in the expression of Hes1 but not Hes5. In addition, Wang et al. (1998) showed the upregulation of Hes5 but not Hes1 when the Notch receptor was activated with Delta in oligodendrocyte precursor cells. These studies indicate that there are transcriptional mechanisms in place that enable Notch1 to selectively upregulate only some of its effectors and that this selectivity is context dependent. Our results suggest that p107 could be involved in a transcriptional regulation mechanism that is specific to Hes1 and not Hes5 in neural precursor cells from the telencephalic germinal zone.

### ***P107 and E2F4 bind to the same E2F binding site on the Notch1 regulatory sequences***

The Notch1 promoter and gene contains several E2F binding sites. P107 was found to bind to an E2F binding site in the first intron of the Notch1 gene (Vanderluit et al, 2004). Using chromatin immunoprecipitation we showed that E2F4, the preferential binding partner of p107, also binds to the same site in the first intron of the Notch1 gene. Taking into account the increased levels of Notch1 transcript in p107-/- brains and the fact that p107 and E2F4 are known to form a repressor complex at E2F regulatory sequences

(Ginsberg et al., 1994), this strongly suggests that p107 is negatively regulating the transcription of Notch1 in neural precursor cells through E2F4.

Very little is known about the transcriptional regulation of the Notch receptors or their ligands with the exception of Jagged (Bash et al., 1999; Sasaki et al., 2002). The results from the present study are unique in terms of their contribution to our understanding of the regulation of the Notch pathway. Mechanisms that regulate Notch pathway transcription will be an important focus of future research in the field.

### *P107 negatively regulates Hes1 transcription.*

The Hes1 promoter was examined using Genomatix promoter analysis software (MatInspector), which located three putatitive E2F binding sites. Chromatin immunoprecipitation was used to determine if there was E2F binding activity at any of the putative sites. These studies revealed E2F3 was binding to sites 1 and 3 and that E2F4 was binding to site 3 and weakly to site 2. The luciferase reporter assay was employed to assess whether Hes1 promoter activity was responsive to any of the pocket proteins. Our results showed that p107 significantly repressed Hes1 promoter activity, however, Rb had no effect. It was previously determined that removal of Rb from neural precursors had no effect on self-newal (Vanderluit et al., 2004). Our results showing that the Hes1 promoter is not responsive to Rb is consistent with our laboratory's previous findings that stem cell self-renewal regulation is a unique function of p107 and not Rb.

Having shown that both E2F3 and E2F4 bind to the putative E2F binding sites on the Hes1 promoter using chromatin immunoprecipitation, we next used the luciferase reporter assay to determine whether p107 could repress promoter activity through either of these E2F family members. We found that both E2F3 and E2F4 significantly repressed the promoter individually. The luciferase reporter assay was carried out in HEK 293 cells, which have been transformed with adenovirus type 5 (Graham et al., 1977), and express the viral protein E1A. E1A interacts with the pocket domain of the pocket protein family at the LXCXE binding site, and is responsible for disassembling the pocket protein-E2F complex (Lee et al., 2002). Thus, with E1A being constitutively expressed in this cell line, one would expect most Rb family activity to be inhibited. Since we are still seeing E2F family repression without co expression of a pocket protein family member, this would suggest that the E2Fs are either interacting with any endogenous free p107 that has not yet interacted with E1A or the overabundance of exogenous E2F protein is out-competing and/or displacing E1A to form a complex with endogenous p107.

Having located three putative E2F binding sites on the Hes1 promoter, and determining that the promoter is responsive to E2F and p107, the next step would be to assess which of the three sites are required for p107 mediated repression of Hes1 promoter activity. One way to achieve this would be to construct plasmids containing the Hes1 promoter with different combinations of the three E2F binding sites deleted and used in the luciferase reporter assay. When the construct missing the essential E2F binding site(s) for p107 mediated repression is cotransfected with p107, there should be no repression of promoter activity.

The luciferase reporter assay is a powerful technique to assess whether a promoter is responsive to certain proteins, and can give important information in terms of how transcription is being regulated. However, this experiment also has its limitations, which must be taken into account when interpreting the results. For example, it is an artificial system, where transformed cell lines are being used instead of primary cells. In addition, cells are being transfected with an extremely large quantities of DNA and subsequently forced to overexpress certain proteins at levels that would never been seen *in vivo.* The method of transfected DNA is also episomal and does not integrate into the chromatin. All of these conditions are not representative of a physiological state. Despite these limitations of the luciferase reporter assay, it is never the less very useful and can reveal important information concerning promoter regulation when taken in the context of results from other supporting experiments. For example, our results including assessment of Hes1 mRNA and protein in the presence and absence of p107 as well as chromatin immunoprecipitation support our luciferase assay data.

### ***Hes1 protein levels are not upregulated in E2F3-/- or E2F4-/- neurospheres.***

Following up results from the chromatin immunoprecipitation experiment that showed both E2F3 and E2F4 bound to the Hes1 promoter, and results from the luciferase reporter assay that showed both E2F3 and E2F4 could repress the activity of the Hes1 promoter, we used western blot analysis to determine if Hes1 protein levels were upregulated in either E2F3-/- or E2F4-/- neural precursor cells. We were interested to determine if p107 requires either one of these E2Fs to mediate Hes1 transcriptional regulation. Our results showed that the protein levels of Hes1 were not deregulated in the absence of either E2F3 or E2F4 in neural precursors. This suggests that neither E2F3 nor E2F4 alone are essential for p107 mediated repression of Hes1 transcription. It is possible that in the absence of E2F3b the remaining repressive E2Fs, E2F4 and E2F5, are compensating for the loss of E2F3b. Likewise it is possible that E2F3b and E2F5 are compensating for the loss of E2F4. For instance E2F4 and E2F5 have been found to have significantly overlapping functions. Animals that have E2F4 or E2F5 deleted are viable (Humbert et al., 2000; Lindeman et al., 1998), however, when both E2F4 and E2F5 are deleted, double null embryos are non-viable (Gaubatz et al., 2000). This shows that both E2F4 and E2F5 have overlapping functions and can therefore compensate for one another to some extent.

All of the repressive E2Fs would have to be removed to determine if p107 was mediating the Hes1 transcription through E2F. One way this could be achieved, in addition to the previously mentioned deletion construct experiment, would be to cotransfect a dominant negative form of DP1 (DN-DP1) along with p107 and E2F3 or E2F4 in the luciferase assay. A DN-DP1 was constructed by Wu et al. (1996), whereby the DNA binding domain of DP1, the binding partner of E2F, was deleted. They showed that DN-DP1 inhibited E2F mediated transactivation. If p107 is repressing Hes1 transcription through the repressive E2Fs then inactivation of the E2F family members by DN-DP1 should relieve p107 mediated repression of Hes1 promoter activity.

### ***P107 regulates neural stem cell self-renewal through interactions with the Notch1-Hes1 signalling pathway.***

Previous studies from our laboratory showed an increase in neural precursor self-renewal and an upregulation of the Notch signalling pathway in p107-/- embryos (Vanderluit et al., 2004). We next wanted to determine if the observed upregulation of the Notch1-Hes1 signalling pathway was responsible for the increased neural precursor pool in p107-/- embryos. To test this, we crossed p107 deficient mice with Hes1 deficient mice to obtain double null p107-/-; Hes1-/- embryos, and assessed the neural precursor pool in these embryos using the primary neurosphere assay. Our results showed that deletion of Hes1, which is downstream of the Notch1 signalling pathway, restored the number of neurosphere forming cells in p107-/- embryos to wildtype levels. This demonstrates that p107 is regulating the neural precursor pool through the Notch1-Hes1 signalling pathway. Taking into account the presence of E2F binding sites on the Hes1 promoter and the results showing that p107 represses Hes1 promoter activity, this further suggests that p107 is negatively regulating the neural precursor pool, by repressing the transcription of Hes1.

Since there are currently no markers that differentiate between neural stem cells and the more restricted progenitors in the brain, the neurosphere assay is an important tool for identifying and studying stem cells. There are, however, drawbacks to using this assay. For instance, it is an *in vitro* assay where higher than physiological levels of growth factors are present in the culture media. This has lead researchers to question whether the stem cells, and consequently the properties that we are measuring with this assay, such as self-renewal, are changing in these culture conditions. To control for this possibility, we used wildtype cells as a control and examined the changes in neural precursor pool of the Hes1-/-; p107-/- mice compared to that of wildtype animals.

In light of the limitations of the neurosphere assay, it is important to follow up our *in vitro* results with *in vivo* experiments. For example the neural stem cell pool could be assessed in adults using BrdU injections. In adults the stem cell population cycles very slowly, about every 15 days in contrast to the progenitor population which cycles every 12.7 hours (Morshead et al., 1992; Reynolds et al., 1992a; Morshead et al., 1994; Morshead et al., 1998). With a series of BrdU injections administered over 12 hours all of the cycling progenitors and stem cells will incorporate BrdU during the injections. The injected animals are then sacrificed four weeks later. At this time the faster cycling progenitors will have cycled several times and will have diluted out the BrdU label, however, the slowly cycling stem cells will have divided a few times and will still be strongly labelled. These strongly labelled stem cells can be counted to give an assessment of the stem cell pool. This BrdU labelling experiment was previously used to assess the stem cell pool in p107-/- adults and it was found that the p107-/- adults had a larger stem cell pool compared to wildtype littermates (Vanderluit et al., 2004). Following up these results, and the results from the present study, we could use the BrdU labelling experiment to determine if p107 is regulating the stem cell pool through Hes1 *in vivo*. Hes-/- animals die by E14.5, so only Hes +/- adult animals could be assessed. Hes +/-;p107 +/- mice could be crossed together to produce both wildtype and Hes+/-; p107-/- animals. By reducing the gene dosage of Hes1 by half, we would expect that the stem cell pool would also be reduced compared to p107 -/- animals as is seen in the neurosphere assay.

### *Future Directions*

It has previously been shown that the Notch pathway is context dependent and that different tissues and cell types activate the Notch pathway for different outcomes (Kim et al., 1996; Deftos et al., 1998; Kumano et al., 2001; Crowner et al., 2003). It would be interesting to determine if regulation of the Notch1-Hes1 signalling pathway by p107 is unique to the neural precursor population or if it is present in other systems. LeCouter et al. (1998b) found that p107-/- mice had significantly more myeloid progenitors compared to wildtype. It is tempting to speculate that this increase in progenitors in the absence of p107 is due to an upregulation of the Notch1-Hes1 signalling pathway .

Three putative E2F binding sites were found on the Hes1 promoter using the promoter analysis software, MathInspector. The presence of E2F sites suggested that Hes1 transcription was being regulated by the pocket protein family through the E2F family of transcription factors. This was further supported by studies using chromatin immunoprecipitation, which showed that there was E2F binding at these sites and that the promoter was responsive to E2F3, E2F4, and p107. It would be interesting to examine the Notch1 and Hes1 promoter and gene sequences of other species such as drosophila, and human to determine if they also possess E2F binding sites. The presence of E2F sites in the Hes1 promoter of other species as diverse as fly to human would indicate that the p107 mechanism of Hes1 transcriptional repression was evolutionarily conserved, and therefore important.

In light of the fact that both Notch1 and Hes1 contain E2F binding sites, it would be interesting to examine the promoters of other genes involved in the Notch1-Hes1 signalling pathway, such as the Notch1 ligands, Delta-like and Jagged, and the NICD transcriptional coactivator RBP-Jk, to see if they also contain E2F binding sites. The presence of E2F binding sites on several members of the Notch pathway would suggest that this pathway is being regulated by p107 at several different levels.

It has recently been discovered that Hes1 directly represses the transcription of p27Kip1, a cyclin-dependent kinase inhibitor, in HeLa cells (Murata et al., 2005) and since mice lacking p27Kip1 show an increase in their neural progenitor population (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996), this suggests a mechanism by which Hes1 promotes the proliferation of progenitors. Our results showing that p107 regulates the neural precursor pool by repressing Hes1 transcription, in turn suggests that p107 is regulating p27Kip upstream through repression of Hes1 to mediate neural precursor proliferation and regulate neural precursor number. It would be interesting to examine the levels of the p27Kip and other cyclin-dependent kinase inhibitors such as p19Arf and p16INK4a in p107-/- neurospheres to support this.

### ***Conclusions***

These studies have shown that p107 represses the transcription of Hes1 in neural precursors, and that in the absence of p107, upregulation of the Notch1-Hes1 signalling pathway is the mechanism responsible for the enhanced neural precursor pool. This is an important physiological interaction whereby cell cycle genes may directly impact the course of differentiation.

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