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

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

Introduction

Cell cycle genes and specifically those genes that regulate the G1/S transition have been shown to play an important role in regulating the neural precursor population. Members of the cyclin-dependent kinase inhibitor (CDKI) family have received much of the attention. CDKIs, p21Cip1, and p27Kip1 negatively regulate embryonic and adult neural precursor proliferation (Doetsch et al., 2002; Kippin et al., 2005). Bmi-1 promotes self-renewing cell division in both hematopoietic and neural precursors through the transcriptional repression of CDKIs, p16Ink4a, and p19Arf (Molofsky et al., 2003, 2005). However, cell cycle regulators impacting the neural precursor population are not only restricted to CDKIs (McClellan and Slack, 2006). We have recently shown that the Retinoblastoma (Rb) family member p107, an inhibitor of the cell cycle G1/S transition, negatively regulates the neural precursor pool in the developing and adult brain by regulating self-renewal (Vanderluit et al., 2004).

p107 has been shown to function by interacting with E2F transcription factors (preferentially E2F4) to repress the transcription of genes required for cell cycle progression (Stevaux and Dyson, 2002). Distinct from other Rb family members, p107 is only expressed in cycling neural precursor cells in the ventricular zone (VZ; Jiang et al., 1997).

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

In this study, we demonstrate that the p107-mediated regulation of neural precursor number occurs through the repression
As the increased number of neural precursor cells in p107-deficient mice could be the result of an increased total population or an enhanced rate of cell division, we assessed the proliferative index in embryonic and adult wild-type and p107−/− mice. Double immunohistochemistry for BrdU and PCNA was performed on adult brains after 10.5 h of cumulative BrdU labeling and on embryonic brains after a 2-h pulse of BrdU. The number of cells that entered S phase (BrdU+ cells) of Hes1 transcription. Hes1 is elevated in p107-deficient brains. Loss of a single Hes1 allele restores the neural precursor population to wild-type levels both in vitro and in vivo. Despite the expanded progenitor population, p107-deficient brains exhibit a reduction in the number of cortical neurons that cannot be accounted for by apoptosis. Short- and long-term BrdU labeling studies revealed a striking defect in the rate at which p107-null progenitors commit to a neuronal fate. Loss of a single Hes1 allele on a p107-null background rescues the number of neurons born during cortical development. Together, these results identify that the mechanism by which p107 regulates both neural precursor self-renewal and differentiation is through regulation of the Notch–Hes1 signaling pathway. In summary, we identify a novel function for p107, a cell cycle regulatory protein, in controlling the onset of differentiation.

Results

p107 regulates the size of the neural precursor population

To determine the temporal requirement for p107 in regulating the neural precursor population, we counted the number of proliferating precursors in the brains of mice at three different ages: in adults and in embryos at embryonic days (E) 10.5 and 13.5. Using antibodies to label cells in the cell cycle (proliferating cell nuclear antigen [PCNA], which labels cells in all phases of the cell cycle; phosphohistone H3 [PH3], which labels cells in M phase; and BrdU, which gets incorporated during S phase), we demonstrate an increase in the proliferating precursor population in p107-null mice. Adult p107-null mice have a 50% increase in the number of precursors, as demonstrated by both cumulative BrdU labeling of proliferating progenitor cells and PCNA immunostaining (Fig. 1, a–d). Similarly, at both embryonic time points E10.5 and 13.5, p107-null embryos had more precursor cells (Fig. 1, e and f). The difference is most pronounced in adult mice when proliferation rates are slower, with a cell cycle time of ~12.7 h (Morshead and van der Kooy, 1992). These studies demonstrate that p107 mutants have an expanded precursor population.

As the increased number of neural precursor cells in p107-deficient mice could be the result of an increased total population or an enhanced rate of cell division, we assessed the proliferative index in embryonic and adult wild-type and p107−/− mice. Double immunohistochemistry for BrdU and PCNA was performed on adult brains after 10.5 h of cumulative BrdU labeling and on embryonic brains after a 2-h pulse of BrdU. The number of cells that entered S phase (BrdU+ cells)
was compared with the total proliferating population (PCNA-expressing cells) in wild-type and p107\(^{-/-}\) mice. A more rapid cell cycle time in p107 knockouts would result in a greater percentage of cells in S phase (Cayre et al., 2005). In adult wild-type mice, 78.2 ± 1.4% of the proliferating population incorporated BrdU, whereas in p107\(^{-/-}\) mice, 78.0 ± 1.9% were BrdU positive (Fig. 1 g). After a 2-h BrdU pulse at E13.5, 53.6 ± 1.8% of wild-type and 51.3 ± 3.2% of p107\(^{-/-}\) cells incorporated BrdU (Fig. 1 h). Thus, comparable proliferative indices in adult and embryonic ages indicate similar cell cycle times. Therefore, the increased number of total progenitor cells at each stage of development represents an overall expansion of the precursor population in p107\(^{-/-}\) mice. This increase in total precursor number is consistent with our previous studies revealing that p107-deficient animals exhibit elevated levels of the Notch1–Hes1 pathway and increased stem cell self-renewal (Vanderluit et al., 2004). As the Notch1 pathway has been shown to play an essential role in stem cell self-renewal (Hitoshi et al., 2002), our expanded precursor population in p107 deficiency is consistent with an increase in the Notch–Hes1 pathway. Therefore, we asked whether the expanded precursor population in p107-deficient mice is the result of the deregulation of Hes1.

**Deregulated Hes1 expression in p107\(^{-/-}\) neural precursors**

Because we have previously demonstrated the deregulation of Notch signaling in p107-deficient neural precursors (Vanderluit et al., 2004), we examined the expression of Notch targets Hes1 and Hes5 in p107 deficiency. Hes1 and Hes5 are basic helix-loop-helix transcription factors that act downstream of Notch to regulate neural precursor self-renewal (Kageyama et al., 2005). In situ hybridization revealed elevated levels of Hes1 transcript in cells of the VZ in p107\(^{-/-}\) mice at E14.5 (Fig. 2, a and b). Quantitative real-time RT-PCR further demonstrated increased Hes1 mRNA in embryonic p107-deficient cortices (Fig. 2 c). In contrast to Hes1 expression, no difference in Hes5 mRNA was detected by in situ hybridization or real-time RT-PCR (Fig. 2, d–f). An examination of Hes1 protein by Western blotting also revealed enhanced expression in neurospheres derived from p107\(^{-/-}\) embryos (Fig. 2 g). Although both Hes1 and Hes5 are Notch1 targets, only Hes1 is deregulated in p107\(^{-/-}\) mice. The selective deregulation of Hes1 expression leads us to question whether p107 could regulate Hes1 transcription.

**p107 represses Hes1 promoter activity**

Because in situ hybridization and real-time RT-PCR reveal that Hes1 is deregulated in p107\(^{-/-}\) neural precursors, we questioned whether p107 could regulate Hes1 transcription. To determine whether the Hes1 promoter is responsive to p107, we used a luciferase promoter assay. The Hes1 promoter (−1,500 bp) was inserted into a pGL3-Basic reporter vector containing the luciferase gene (B-Hes1; Fig. 3 a). This construct was transfected into HEK 293A cells along with 3 μg of a p107 expression vector or Rb expression vector as a control. Cotransfection of 3 μg p107 resulted in a 2.5-fold reduction in Hes1 promoter activity (Fig. 3 b). This repression was dose dependent because a further increase in p107 (10 μg) resulted in a >10-fold repression. In contrast, protein Rb (pRb) did not repress Hes1 promoter activity. These results demonstrate that p107 represses Hes1 promoter activity.

Because p107 regulates transcription by interacting with E2F transcription factors, we asked whether E2Fs were required for p107-mediated repression. The Hes1 promoter was analyzed, and three putative E2F-binding sites (BSs [E2F-BSs]) were located at positions −560, 161, and 398 bp relative to the transcription start site (Fig. 3 a). To test whether E2Fs were required for p107-mediated repression, we deleted all three BSs from the Hes1 promoter (Hes1-3BS). Cotransfection of the Hes1-3BS construct with the p107 expression vector resulted in pronounced repression of luciferase activity (Fig. 3 c). These results demonstrate that p107-mediated repression of the Hes1 promoter occurs indirectly, independent of these E2F sites. Nevertheless, our results showing decreased levels of Hes1 transcript and protein combined with p107-mediated repression of the Hes1 promoter demonstrate that p107 is required in the neural precursor population for the repression of Hes1.

**Figure 2. Hes1 expression is deregulated in p107\(^{-/-}\) precursor cells.** (a and b) In situ hybridization revealed higher levels of Hes1 mRNA in the VZ of E14.5 p107\(^{-/-}\) mice versus wild-type littermates. (c) Elevated levels of Hes1 mRNA were also demonstrated by real-time RT-PCR on whole cortices from E12.5 wild-type (n = 4) and p107\(^{-/-}\) (n = 4) embryos. (d–f) In contrast, no difference was observed in the levels of Hes5 mRNA between wild type and p107\(^{-/-}\). (g) Neuroepithelia from E10.5 wild-type (wt) and p107\(^{-/-}\) mice were cultured as neurospheres. Protein was isolated from the neurospheres for Western blot analysis. A representative Western blot initially probed with an antibody to Hes1 and then reprobed with an antibody to actin to verify equal quantities of protein was loaded per lane. The experiment was performed in triplicate. VZ, ventricular zone; C, cortical plate and IZ. Means were analyzed by a t test with *, P < 0.05. Error bars represent SEM. Bar, 50 μm.
To ask whether p107 regulates the neural precursor population by the repression of Hes1, we interbred p107-deficient mice with animals carrying a null mutation for Hes1. We hypothesized that if p107 regulated neural precursor cells by controlling the levels of Hes1, the loss of one or more alleles of Hes1 in p107-null mice would partially or completely restore the number of neural precursors back to wild-type levels. Conversely, if p107 acted through an independent pathway, store the expanded precursor population to wild-type levels. Together with the demonstration that p107 represses Hes1 gene expression, these results demonstrate that the expanded neural precursor population in p107−/− embryos results from the derepression of Hes1.

p107 regulates the neural precursor population in vivo through the repression of Hes1

We next questioned whether p107 controlled the size of the neural precursor population in vivo through the repression of Hes1. As a result of the embryonic lethality of double mutant mice, we asked whether the loss of a single Hes1 allele could restore the number of neural precursor cells in p107-null mice to wild-type levels. In adult mice, the precursor population was labeled with BrdU. Counts of BrdU-positive cells in adult brains showed that a reduction in Hes1 could restore the number of proliferating precursors in p107-null mice (Fig. 5 a). PH3 immunohistochemistry of the rapidly dividing E10.5 neural precursors demonstrated that loss of a single Hes1 allele in p107-null embryos (Hes1−/−; p107−/−) reduced the number of cells in M phase to levels comparable with the wild type (Fig. 5 b). Similarly, a 2-h BrdU pulse in E13.5 embryos also revealed a reduction in the number of cells in S phase in p107−/− embryos lacking a single Hes1 allele (Fig. 5 c). Loss of a single Hes1 allele in p107−/− mice restored the number of proliferating cells to wild-type levels at all three developmental time points. In contrast, loss of a single Hes1 allele on a wild-type background (Hes11/+) did not result in a reduction in the number of proliferating cells (Fig. 5 c). These results demonstrate that p107 regulates the neural precursor population by controlling the levels of Hes1 in progenitor cells.

Figure 3. p107 represses Hes1 promoter activity. (a) Diagram of the Hes1 promoter (−1,500 bp) containing three putative E2F-BSs at positions −560, 161, and 398 bp relative to the transcriptional start site. (b) Luciferase assay of HEK 293A cells transfected with the pGL3B-Luc Hes1 promoter constructs and 3 μg of expression vectors containing either Rb or p107. Rb has no effect on Hes1 promoter activity, whereas 3 μg p107 represses Hes1 promoter activity by a factor of 2.5, and 10 μg p107 represses 10-fold. (c) Transfection of the pGL3B-Luc Hes1-3xBS promoter (E2F-binding mutant) with p107 still resulted in the repression of promoter activity, suggesting that p107 represses Hes1 transcription through an E2F-independent mechanism. Experiments were performed in triplicate. Means were statistically analyzed by one-way analysis of variance followed by Tukey’s individual comparison of the means. Error bars represent SEM. *, P < 0.05.

Figure 4. Loss of Hes1 restores the number of neural precursors to wild-type levels in p107−/− embryos. Quantification of neurospheres revealed that p107−/− cultures produced significantly more neurospheres than all of the other genotypes, including Hes1−/−; p107−/− and Hes1−/−; p107−/−. Therefore, a reduction in Hes1 expression in p107−/− mice restores the number of neural precursors back to wild-type levels (wild type, n = 3; p107−/−, n = 3; Hes1−/−; p107−/−, n = 6; Hes1−/−; p107−/−, n = 5; Hes1−/−; p107−/−, n = 3; Hes1−/−; p107−/−, n = 4; Hes1−/−; p107−/−, n = 4). Means were statistically analyzed by one-way analysis of variance followed by Tukey’s individual comparison of the means. Error bars represent SEM. *, P < 0.05.
p107 deficiency leads to a decreased number of mature neurons

The expanded neural precursor numbers in p107−/− mice lead us to question whether the number of cortical neurons was also affected. Specifically, we asked whether the expanded precursor population leads to increased neurogenesis. Immunohistochemistry with NeuN, a marker for mature central nervous system neurons, revealed a significant reduction (P < 0.05) in the number of NeuN-expressing cells in p107−/− cortices (Fig. 6, a–c). The decreased number of cortical neurons is further demonstrated by a reduction in the overall size/ thickness of the cortex (Fig. 6, d and f), whereas measurements of the VZ did not reveal any differences between p107−/− mice and wild type at E18.5 (Fig. 6 e). Because p107-deficient mice exhibit a reduction in the total number of cortical neurons despite an expanded progenitor pool, we questioned whether p107 was required for the survival of cortical neurons.

To determine whether an increase in cell death was responsible for the reduction in cortical neuron numbers in p107-deficient brains and at what stage in development cells were dying, we counted the number of apoptotic cells in the VZ/SVZ, the intermediate zone (IZ), and in the cortical plate and marginal zone. Apoptotic cells were identified by immunohistochemistry for active caspase-3 and Hoechst nuclear staining in E13.5 embryonic brains (Fig. 7, a–f). The number of apoptotic cells in the brains of wild-type and p107−/− mice was low, with less than one cell per 1,000-μm² area (Fig. 7 h). As a result of the low frequency of apoptotic cells, counts were performed in 12 sections throughout the telencephalon in both left and right hemispheres, and the total number of apoptotic cells was compared between genotypes (Fig. 7 g). A twofold increase in the number of apoptotic cells was observed only in the VZ/SVZ of p107-null brains, whereas no differences were observed in the IZ or cortical plate/marginal zone. An increase in cell death in the p107 mutant VZ/SVZ suggests that newly committed neurons may be dying in the VZ before they initiate migration.

To identify at which stage in progenitor cell development cells were dying, double immunolabeling was performed with antibodies to active caspase-3 and Nestin (progenitor cell marker), doublecortin (migratory neuroblast), and βIII-tubulin (Tuj1, an early neuronal marker). Because the increase in apoptotic cells is only in the VZ/SVZ, we counted the double-labeled cells in this region. At E13.5, most cells were double labeled with active caspase-3/Nestin (Fig. 7, a–f), suggesting that p107 affects the survival of uncommitted progenitor cells. No double labeling was detected with doublecortin or Tuj1 in any of our samples, ruling out the possibility that p107 deficiency results in enhanced cell death in newly committed neuroblasts. As apoptosis progresses, dying cells will lose their cell type–specific markers; thus, some pyknotic cells did not colabel with Nestin or any other marker examined. The colabeling of apoptotic cells with caspase-3 and Nestin is consistent with the interpretation that cell death is occurring in uncommitted progenitor cells in the
Neuronal commitment. To address this question, we performed a 24-h BrdU incorporation to measure the rate of neuronal commitment. Pregnant dams were injected at E13.5, embryos were collected 24 h later at E14.5, and the number of strongly labeled BrdU-positive cells were counted (i.e., cells that underwent terminal mitosis at the time of injection). In addition, sections were double stained with PCNA to show that these cells are no longer cycling. Double labeling with BrdU and PCNA revealed that most BrdU-positive cells within the SVZ and IZ were no longer expressing PCNA, indicating that they were newly postmitotic (Fig. 8, d–f). Cell counts of this newly postmitotic population revealed a two-fold reduction in p107-deficient brains compared with wild-type controls (Fig. 8, f and i). These results demonstrate that there is a decrease in the number of newly postmitotic cells leaving the VZ in p107-deficient animals.

We hypothesized that a decrease in the number of newly postmitotic cells migrating out of the VZ/SVZ in p107-deficient mice indicates that p107 may be required for the regulation of neuronal commitment. To test this possibility, we performed the aforementioned 24-h BrdU commitment assay followed by double labeling with BrdU and doublecortin to identify migrating neuroblasts or BrdU and Tuj1, an early panneuronal marker in the brain, as a neuronal fate, resulting in a twofold reduction in cortical neurons in p107-deficient brains compared with wild-type controls (Fig. 8, f and i). These findings support a model whereby fewer p107-deficient progenitor cells commit to a neuronal fate, resulting in a twofold reduction in cortical neurons in the brain at E18. In summary, our results reveal that p107 is required for neuronal commitment and promotes the decision to exit the progenitor pool and commit to a neuronal fate.

Because our results show that Hes1 is involved in p107-mediated neural precursor self-renewal and Hes1 functions to maintain the neural precursor population by repressing the expression of proneural genes (Sasai et al., 1992; Ishibashi et al., 1995), we asked whether deregulated Hes1 could account for the defect in neurogenesis in p107-null mice. The loss of a

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Figure 7. Reduced neuronal numbers is not the result of enhanced apoptosis in the cortical plate. (a–f) To determine whether p107 was required for the survival of postmitotic neurons in the cortical plate, we counted the number of apoptotic cells in the developing cortex of E13.5 embryos. Apoptotic cells were identified by active caspase-3 immunohistochemistry and Hoechst nuclear staining. (g and e) Within the SVZ/VZ, colabeling of active caspase-3 and Nestin identified the majority of dying cells as uncommitted progenitors. (g) Total apoptotic cells were counted across 12 representative sections in both left and right hemispheres through the telencephalon. Cell counts revealed no increase in apoptosis in the VZ/SVZ of p107−/− embryos but not in the developing cortex (wild type, n = 4; p107−/−, n = 4). (h) The number of apoptotic cells per 1,000-μm length along the ventricle and up through to the pial layer showed an extremely low frequency of cell death per counting area. Mean counts were assessed by a two-way analysis of variance followed by Bonferroni’s individual comparison of means. CP, cortical plate; MZ, marginal zone. Error bars represent SEM. *, P < 0.05. Bar, 25 μm.

VZ/SVZ. The increase in apoptosis in the VZ/SVZ did not reveal any death of newly committed neurons. In conclusion, these studies show that the absence of p107 results in the increased cell death of Nestin-expressing progenitor cells.

p107 controls neural progenitor commitment to a differentiated fate in vivo

As the increased apoptosis in the VZs of p107-null mice is caused by the death of uncommitted Nestin-expressing progenitor cells and the rate of cell death was still very low, we questioned whether the absence of p107 may result in a defect in the rate of neuronal commitment. To address this question, we performed neuronal birthdating assays using a BrdU protocol to label cells undergoing terminal mitosis at the time of BrdU injection. Neuronal birthdating is based on the demonstration that neurogenesis occurs between E12 and 17, during which cohorts of neural precursors are born (neuronal commitment) at distinct time points and migrate out of the VZ to form the layers of the cortex (Caviness, 1982; Caviness et al., 1995; Takahashi et al., 1999).

A single BrdU injection labels all cells in S phase, but only neural precursors undergoing terminal mitosis retain the BrdU label. Therefore, BrdU birthdating provides a quantitative analysis of cells that commit to a neuronal fate, undergo terminal mitosis, and migrate to their ultimate destination in the cerebral cortex. Accordingly, pregnant dams were injected with BrdU at E13.5 (Takahashi et al., 1999), the time at which deep layer cortical neurons are generated. Embryos were collected 5 d after injection at E18.5. BrdU cell counts revealed that p107−/− mice (36 ± 5; n = 5) had a dramatic twofold reduction in the number of neurons that were born at injection time (E13.5) and reached the cortical plate by E18.5 relative to wild-type littermates (68 ± 9; n = 4; Fig. 8, a–c). These results show that there is a decrease in the number of neurons reaching the cortical plate in p107-deficient brains.

Because our examination of cell death did not reveal a loss of newly generated neurons in the cortical plate, we asked whether the reduced neuronal numbers in p107-deficient mice was caused by fewer neurons born at E13.5 (the time of BrdU injection). To address this question, we performed a 24-h BrdU incorporation to label cells undergoing terminal mitosis at the time of injection (Fig. 8, d–f). Cell counts of this newly postmitotic population revealed a two-fold reduction in p107-deficient brains compared with wild-type controls (Fig. 8, f and i). These results demonstrate that there is a decrease in the number of newly postmitotic cells leaving the VZ in p107-deficient animals.

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Bars: (a–e) 100 μm; (g, h, j, k, m, and n) 25 μm.

Figure 8. **Defective differentiation in p107−/− neural precursors.** Long-term BrdU birthdating analysis (E13–18) showed a reduction in the number of cells exiting the VZ and arriving in the cortical plate. Pregnant females received an intraperitoneal injection of 20 mg/kg BrdU at gestation day 13.5 (E13.5). Embryos were collected 5 d later at E18.5, and BrdU− cells were counted in the developing cortices. (a–c) p107+/− embryos had significantly fewer BrdU− cells in the cortex in contrast to wild-type embryos (wild type, n = 4; p107−/−, n = 4). (d–f) To assess the number of neurons born at E13, short-term BrdU birthdating (E13–14) was performed with pregnant females receiving an intraperitoneal injection of 20 mg/kg BrdU at E13.5, and embryos were collected 24 h later at E14.5. (d and e) Strongly labeled BrdU− cells were primarily located in the IZ and SVZ. (f) Quantification of the number of strongly labeled BrdU− cells revealed a significant reduction in p107−/− deficient brains. (g–i) Double labeling of BrdU and PCNA was performed to assess whether cells had undergone terminal mitosis and exited the cell cycle (i.e., BrdU−/PCNA− cells; arrows). (j–l) Double labeling of BrdU and DCX revealed a significant reduction in p107−/− deficient brains. (j–l) Double labeling of BrdU and Tuj1 revealed a significant reduction in p107−/− deficient brains. (m–o) Double labeling of BrdU and TuJ1 revealed a significant reduction in p107−/− deficient brains. (m–o) Double labeling of BrdU and TuJ1 revealed a significant reduction in p107−/− deficient brains.

Discussion

In this study, we identify a novel role for p107 in regulating the transition from proliferating neural progenitor cell to committed neuroblast. During neural development, p107 regulates the size of the neural precursor pool by limiting self-renewal capacity and promoting the transition from progenitor cell to committed neuroblast. Furthermore, we show that the mechanism by which p107 regulates the neural precursor pool is through repression of the Notch–Hes1 pathway.

The cell cycle protein p107 promotes neural precursor self-renewal through the repression of Hes1

In this study, we demonstrate that p107 regulates neural precursor self-renewal through the repression of Hes1. Our previous work has shown that p107-deficient neural precursors have an enhanced self-renewal capacity and that Notch1 and Hes1 are up-regulated (Vanderluit et al., 2004). Because we observed an up-regulation of only Hes1 and not Hes5, we questioned whether p107 might be acting at multiple levels along the Notch–Hes1 pathway. Although promotor analysis reveals that Hes1 repression is indirect, likely resulting from Notch activation, these experiments set the rationale for using the Hes1 mutant mice to reduce signaling through the Notch1 pathway. By interbreeding p107 and Hes1 mutant mice, the defect found in p107 mutant is rescued.
The loss of one or more Hes1 alleles in p107-deficient mice restores both the size of the neural precursor pool to wild-type levels and the differentiation defect. These results identify a novel mechanism whereby p107, a cell cycle protein, regulates the neural precursor population through the repression of Hes1, a gene that inhibits differentiation by repressing proneural gene transcription. Although previous studies have shown that cell cycle inhibitors such as the CDKIs (p16Ink4a, p18Ink4c, p19Arf, p21Cip1, and p27Kip1) negatively regulate neural precursor proliferation, they function by direct inhibition of the cell cycle machinery (cyclin kinases; Cheng et al., 2000; Doetsch et al., 2002; Molofsky et al., 2003, 2005; Yuan et al., 2004; Kippin et al., 2005). In contrast, p107 regulates the transcription not only of cell cycle genes but also genes that impact the onset of differentiation, such as Hes1. Our studies support a novel concept whereby the role of cell cycle genes such as p107 extends beyond regulation of the cell cycle machinery and directly impacts the onset of differentiation in the developing nervous system.

**p107 promotes progenitor commitment to a neuronal fate**

During cortical development, progenitor cells in the VZ commit to a neuronal fate, undergo terminal mitosis, and commence migration into the cortical plate, where they undergo differentiation. Previously, we demonstrated that p107 regulates neural precursor numbers by limiting self-renewal (Vanderluit et al., 2004). In the present study, we extend these findings to show that p107 has an additional function in controlling this population by regulating commitment to a neuronal fate. p107-deficient mice have an overall reduction in the number of cortical neurons, resulting in considerably smaller brains despite an expansion of the neural precursor population. This was not caused by the increased cell death of committed neurons because no difference in the number of apoptotic cells in the p107-deficient cortical plate was found. Although an increase in apoptosis was observed in the VZ/SVZ of p107-deficient mice, double labeling revealed that these cells coexpressed Nestin, indicating that apoptosis was elevated in uncommitted progenitor cells. Consistent with this, there was no double labeling of VZ/SVZ apoptotic cells with doublecortin or TuJ1, which are markers for newly committed neurons. There is the possibility that newly committed SVZ progenitors may undergo apoptosis before the expression of early commitment markers, but the frequency of apoptotic cells is very low, making this interpretation unlikely.

In further support of a defect in neuronal commitment, we performed a 24-h (E13–14) BrdU labeling analysis, which revealed a substantial reduction in the number of newly committed neurons in p107-deficient brains (Fig. 8). These findings support our long-term birthdating (E13–18) study that demonstrated a twofold decrease in the number of cortical neurons (Fig. 6), further supporting a model whereby fewer neurons are born during the time that neurogenesis takes place in the developing p107-deficient cortex. This demonstrates that p107 promotes progenitor cell commitment to a neuronal fate. Whereas in p107-deficient mice, expanded precursor populations have been identified in neuronal, myeloid, and adipocyte lineages (LeCouter et al., 1998; Vanderluit et al., 2004; Scime et al., 2005), our studies reveal a striking defect in the ability of progenitor cells to commit to a neuronal fate and undergo differentiation. Furthermore, the mechanism underlying this defect is through the deregulation of Hes1, an inhibitor of differentiation that functions by repressing the proneurogenic genes Ngn1, Mash1, and NeuroD (Sasai et al., 1992; Ishibashi et al., 1995). Collectively, these results reveal a novel function for p107 in promoting neural precursor cell commitment to a neuronal fate.

**Rb family members perform distinct roles in neurogenesis**

Collectively with our previous findings (Ferguson et al., 2002; Vanderluit et al., 2004), the results of this study reveal temporally sequential functions for Rb family proteins during the course of neural development (Fig. 10). First, p107 is expressed in proliferating neural precursor cells and controls the size of the neural precursor population. It does so by regulating neural precursor self-renewal and commitment to a neuronal fate. In the absence of p107, the progenitor pool is expanded by increased self-renewal and impairment in neuronal commitment. This function is unique for p107 because pRb-null mice do not exhibit an expanded population of neural precursor cells, nor do they show a decrease in neurogenesis (Ferguson et al., 2002). The temporal requirement for pRb occurs at the time of neuronal differentiation. Unlike p107-deficient animals, pRb-null progenitors successfully commit to a neuronal fate but exhibit impairment in migration and differentiation. This is evident by the widespread ectopic mitoses of committed progenitors throughout the developing cortex. Furthermore, committed neuroblasts derived from the ventral populations fail to migrate through their tangential trajectories to reach their final destinations in the dorsal telencephalon (Ferguson et al., 2002, 2005). Thus, as p107 becomes down-regulated, pRb plays an essential role to regulate differentiation and neuronal migration. Once neurons complete terminal differentiation, pRb becomes down-regulated, and p130 becomes the predominant Rb family member (Jiang et al., 1997;
Gill et al., 1998; Callaghan et al., 1999; Ferguson et al., 2000; Yoshikawa, 2000). Recent studies reveal that p130 is highly expressed in postmitotic cells and plays an important role in the regulation of neuronal survival (Liu and Greene, 2001; Liu et al., 2005). Thus, each Rb family protein plays a temporally distinct role that is crucial for normal neural development.

In conclusion, the key finding of this study is that p107, a cell cycle regulatory protein known for its role in controlling the cell cycle machinery, has a novel function in regulating the onset of differentiation. This new role provides insight as to how cell cycle proteins may participate in shaping the developing brain. We also show that deregulation of the Notch1 pathway is mechanistically important because deletion of a single allele of Hes1 rescues the defects found in p107-deficient mice but has no effect on wild-type animals. In summary, we have identified a novel function for the cell cycle regulator p107 in promoting progenitor cell differentiation through regulation of the Notch–Hes1 signaling pathway.

Materials and methods

Mice

Germline p107-null mice were generated previously by LeCouter et al. (1998) and maintained on a mixed 5 V-129 and C57BL/6 background. Germline Hes1-null mice on an ICR background were originally generated by R. Kageyama (Ishibashi et al., 1995). Hes1/p107-deficient embryos were generated by interbreeding heterozygous (Hes1+/−) mice with p107−/− mice. Animals were genotyped according to standard protocols with previously published primers for p107 (LeCouter et al., 1998) and Hes1 (Ishibashi et al., 1995). For embryonic time points, the time of plug identification was counted as E0.5. All experiments were approved by the University of Ottawa’s Animal Care Ethics Committee, adhering to the guidelines of the Canadian Council on Animal Care.

Tissue fixation and cryoprotection

Pregnant dams and adult mice were killed with a lethal injection of sodium pentobarbital. Embryos were dissected and submersion fixed overnight in 4% PFA in 1× PBS, pH 7.4. Adult mice were perfused with 1× PBS followed by cold 4% PFA, and brains were removed. Brains were postfixed overnight in 4% PFA, cryoprotected in 22°C sucrose in 1× PBS, and frozen, and 14-μm coronal sections through the forebrain were collected on SuperFrost Plus slides (Fisher Scientific).

In situ hybridization and quantitative real-time RT-PCR

Nonradioactive in situ hybridization and digoxigenin probe labeling was performed according to previously described protocols (Wallace and Raff, 1999). Antisense riboprobes for Hes1 and Hes5 were generated according to previously published primers for p107 (LeCouter et al., 1998) and Hes1 (Ishibashi et al., 1995). For embryonic time points, the time of plug identification was counted as E0.5. All experiments were approved by the University of Ottawa’s Animal Care Ethics Committee, adhering to the guidelines of the Canadian Council on Animal Care.

Immunohistochemistry and Western blotting

Immunohistochemistry was performed on coronal cryostat sections from embryonic and adult brains with primary antibodies to mouse anti-NeuN (1:100; Chemicon), rabbit antiactin (1:500; BD Biosciences), rabbit anti-anti-PH3 (1:400; Upstate Biotecnology), rat anti-Brdu (1:100; Accurate Chemicals), mouse anti-Brdu (1:100; Becton Dickinson), mouse anti-PCNA (1:300; Vector Laboratories), goat antidoublecortin (1:100; Santa Cruz Biotechnology, Inc.), mouse anti-βIII-tubulin (mouse monoclonal hybridoma supernatant 1:100; Caccamo et al., 1989), and mouse anti-Nestin (1:400; Research Diagnostics). For Brdu bitharding experiments (E13.5–18.5), pregnant dams received a single injection of Brdu at 20 μg/g of body weight; for short-term Brdu incorporation experiments, pregnant dams and adult mice received intraperitoneal injections of Brdu at 100 μg/g of body weight. For Brdu detection, sections were denatured in 2 N HCl at 37°C for 15 min followed by neutralization in 0.1 M Na borate, pH 8.5, for 10 min at room temperature before incubation with the primary antibody. For PCNA detection, sodium citrate antigen retrieval, pH 6.0, was performed on sections before incubation with PCNA antibodies. In all double labeling with Brdu antibodies, denaturation and Brdu immunohistochemistry were performed after the first primary and secondary antibody incubation.

Protein was isolated from cultured neurospheres in lysis buffer, run on a 15% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane as described previously (Ferguson et al., 2000). Immunoblotting was performed with antibodies directed against Hes1 (provided by H. Kitamura, Yokohama City University School of Medicine, Yokohama, Japan; Ito et al., 2000), Hes5 (Chemicon), and actin (Santa Cruz Biotechnology, Inc.). Blots were developed by chemiluminescence according to the manufacturer’s instructions (NEL100; PerkinElmer).

Cell counts and measurements

In the adult brain, BrdU+ cells were counted along the entire length of the ventricular surface (dorsal and ventral) in every 10th section from the rostral crossing of the corpus callosum to the start of the third ventricle and crossing of the anterior commissure with an equal number of sections counted per brain as previously described (Morshead et al., 1998; Vanderluit et al., 2004). PH3+ cells were counted along a 1,000-μm length of the ventricle in three representative sections through the forebrains of E10.5 embryos. In E13.5, 14.5, and 18.5 embryos, Brdu+ cells and/or NeuN+ cells were counted along a 750-μm length of the ventricle up through to the pial surface in four representative regions through the forebrain (Koutmani et al., 2004). Cortical plate, VZ, and cortical mantle measurements were performed on cresyl violet sections of E18 brains from wild type. Triplicate measurements were performed on three representative sections through the forebrain.

Promoter analysis

The Hes1 promoter [1,270 bp sequence], including a 224-bp 3′ sequence of the transcription start site, was amplified from mouse genomic DNA by PCR and inserted into a pG3L3-Basic luciferase reporter construct (forward primer 5′-CGGCCGCCGAGAATACCT-3′; reverse primer 5′-GATGAGTGCAAGGGGAGAGAGTCTC-3′; Promega; Sasaki et al., 1992; Takebayashi et al., 1994). To assess whether promoter activity was affected by p107, the pG3L3-Hes1 construct or the E2F-BS mutant [Hes1-3xBS] was cotransfected with expression vectors for either p107 (pCMV-p107) or Rb (pOK-RB) into HEK 293A cells by standard calcium phosphate precipitation (Storring et al., 1999). 2 μg pMVLacZ was cotransfected with each sample to control for transfection efficiency. 4-methylumbelliferyl-galactoside assay was performed to standardize the transfection reaction, and luciferase activity was assessed according to standard procedures (Fortin et al., 2004). Statistical analysis was performed on the means of three different experiments.

Table 1. Primers for E2F-mutated Hes1 promoter construct

| Forward | Reverse |
|---------|---------|
| Fragment 1 | 5′-ATCGGTACCGCTGCCTCGTT-3′ |
| Fragment 2 | 5′-ATCGGGATCAGCTTTAATCTC-3′ |
| Fragment 3 | 5′-ATCGGCTTCGAGGGTCTAAAATAATCTC-3′ |
| Fragment 4 | 5′-ATCGGCTTCGACCTCGGGGTTGAAAGTG-3′ |

Primers were designed to exclude each of the three putative E2F-BSs, resulting in fragmentation of the Hes1 promoter into four pieces. Restriction sites were incorporated into primer ends to ease promoter reassembly.
Putative E2F consensus sites were identified by MatInspector software (Genomatix; Fig. 3 q). A mutant Hes1 promoter minus all three E2F-binding sequences (Hes1-3xBS) was constructed by linking PCR fragments on each side of the E2F-BSs and inserting them into the pG3B-luciferase construct (Table I).

Neurosphere assay

The neurosphere assay was performed on neuroepithelia from E10.5 embryos as previously described (Reynolds et al., 1992; Tropepe et al., 1999; Vanderluit et al., 2004). Statistical comparisons were performed on the mean number of neurospheres per embryo per genotype.

Microscopy

Sections treated for immunohistochemistry or in situ hybridization were examined by a microscope (Axioskop; Carl Zeiss MicroImaging, Inc.) with standard fluorescence and brightfield/darkfield settings at 5× NA 0.25 or 20× NA 0.50 objectives. Images were captured using a color video camera (Power HAD 3CCD; Sony) with Northern Eclipse software (Empix Imaging). For confocal microscopy, images were captured using a microscope (LSM 510 META; Carl Zeiss Microimaging, Inc.) on an inverted microscope (Axiovert 200M; Carl Zeiss Microimaging, Inc.) with the manufacturer’s integrated digital imaging software. Figures were compiled using Photoshop 6.0 (Adobe). Manipulations of brightness and intensity were made equally to all treatment groups.

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References

Caccamo, D., D.C. Katsetsos, M.M. Herman, A. Frankfurter, V.P. Collins, and J.A. Rubinstein. 1989. Immunohistological pattern of a spontaneous neural teratoma with neuroepithelial differentiation. Neural-associated beta-tubulin as a marker for primitive neuroepithelium. Lab. Invest. 60:390–398.

Callaghan, D.A., L. Dong, S.M. Callaghan, Y.X. Hou, L. Dagnino, and R.S. Slack. 1999. Neural precursor cells differentiating in the absence of Rb exhibit delayed terminal mitosis and deregulated E2F 1 and 3 activity. Dev. Biol. 207:257–270.

Caviness, V.S., Jr. 1982. Development of neocortical afferent systems: studies in human cerebral cortex. J. Neurosci. Res. 6:2620–2634.

Fortin, A., J.G. MacLaurin, N. Arbour, S.P. Cregan, N. Kushwaha, S.M. Callaghan, D.S. Park, P.R. Albert, and R.S. Slack. 2004. The proapoptotic gene SIVA is a direct transcriptional target for the tumor suppressors p53 and E2F1. J. Biol. Chem. 279:28706–28714.

Gill, R.M., R. Slack, M. Kiess, and P.A. Hamel. 1998. Regulation of expression and activity of distinct PRB, E2F, D-type cyclin, and CDK family members during terminal differentiation of P19 cells. Exp. Cell Res. 244:157–170.

Hatakeyama, J., Y. Bessho, K. Katoh, S. Ookawara, M. Fujisaki, F. Guillemot, and R. Kageyama. 2004. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development. 131:5539–5550.

Hitoshi, S., T. Alexson, V. Tropepe, D. Conlon, T.W. Mak, A. Bernstein, and D. van der Kooy. 2002. Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes Dev. 16:846–858.

Ishibashi, M., K. Moriyoshi, Y. Sasai, K. Shiota, S. Nakanishi, and R. Kageyama. 1994. Persistent expression of helix-loop-helix factor HES-1 prevents mammalian neural survival and death by repressing of neural cell proliferation leading to exhaus- tion of their proliferative capacity. Genes Dev. 19:756–767.

Koutman, T., M. Vooijs, M. Kashimoto, T. Takahashi, and R. Kageyama. 2005. Targeted disruption of mammalian hairy and Enhancer of split homolog-1 (HES-1) leads to up-regulation of neural helix-loop-helix factors, premature neurogenesis, and severe neural tube defects. Genes Dev. 19:3136–3148.

Kageyama, R., T. Ohtsuka, J. Hatakeyama, and R. Ohsawa. 2005. Roles of HHLH genes in neural stem cell differentiation. Exp. Cell Res. 306:343–348.

Kippin, T.E., D.J. Martens, and D. van der Kooy. 2005. p21 compromises the relative quiescence of neuroepithelial stem cell proliferation leading to exhaustion of their proliferative capacity. Genes Dev. 19:719–732.

Liu, D.X., and L.A. Greene. 2001. Neuronal apoptosis at the G1/S cell cycle checkpoint. Cell Tissue Res. 305:217–229.

Liu, D.X., N. Nath, S.P. Chellappan, and L.A. Greene. 2005. Regulation of neuron survival and death by p130 and associated chromatin modifiers. Genes Dev. 19:719–732.

MacLaurin, D.S. Park, V. A. Wallace, M. Vooijs, S.K. McConnell, and R.A. Morris. 2002. Notch path- way regulates the neuroendocrine differentiation of fetal mouse pulmo- nary epithelium. Development. 127:3913–3921.

Molofsky, A.V., S. He, M. Bydon, S.J. Morrison, and R. Pardal. 2005. Bmi-1 promotes neural stem cell self-renewal and neural development but not mouse growth and survival by repressing the p16Ink4a and p19Arf senes- cence pathways. Genes Dev. 19:1432–1437.

Morshhead, C.M., and D. van der Kooy. 1992. Postmitotic death is the fate of constitutively proliferating cells in the subependymal layer of the adult mouse brain. J. Neurosci. 12:249–256.

Reynolds, B.A., W. Tetzlaff, and S. Weiss. 1992. A multipotent EGF-responsive neural progenitor cell producing neurons and astrocytes in vitro. Nature 358:13–20.

Rudnicki, M.A. 2005. Rb and p107 regulate preadipocyte differentia- tion of their proliferation capacity. Genes Dev. 19:719–732.

Rudnicki, M.A. 1998. Strain-dependent myeloid hyperplasia, growth defi- ciency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. Mol. Cell. Biol. 18:7455–7465.

Rubinstein, J.A. 1989. Immunohistological pattern of a spontaneous neural teratoma with neuroepithelial differentiation. Neural-associated beta-tubulin as a marker for primitive neuroepithelium. Lab. Invest. 60:390–398.

Rudnicki, M.A. 1998. Strain-dependent myeloid hyperplasia, growth defi- ciency, and accelerated cell cycle in mice lacking the Rb-related p107 gene. Mol. Cell. Biol. 18:7455–7465.

Ruggiero, F., and P.A. Hamel. 1998. Regulation of expression and function of the cell cycle regulatory protein, Rb, in neuronal migration. EMBO J. 17:4381–4391.
Stevaux, O., and N.J. Dyson. 2002. A revised picture of the E2F transcriptional network and RB function. *Curr. Opin. Cell Biol.* 14:684–691.

Storring, J.M., A. Charest, P. Cheng, and P.R. Albert. 1999. TATA-driven transcriptional initiation and regulation of the rat serotonin 5-HT1A receptor gene. *J. Neurochem.* 72:2238–2247.

Takahashi, T., T. Goto, S. Miyama, R.S. Nowakowski, and V.S. Caviness Jr. 1999. Sequence of neuron origin and neocortical laminar fate: relation to cell cycle of origin in the developing murine cerebral wall. *J. Neurosci.* 19:10357–10371.

Takebayashi, K., Y. Sasai, Y. Sakai, T. Watanabe, S. Nakanishi, and R. Kageyama. 1994. Structure, chromosomal locus, and promoter analysis of the gene encoding the mouse helix-loop-helix factor HES-1. Negative autoregulation through the multiple N box elements. *J. Biol. Chem.* 269:5150–5156.

Tomita, K., M. Ishihashi, K. Nakahara, S.L. Ang, S. Nakanishi, F. Guillemot, and R. Kageyama. 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. *Neuron.* 16:723–734.

Tropepe, V., M. Sibilia, B.G. Ciruna, J. Rossant, E.F. Wagner, and D. van der Kooy. 1999. Distinct neural stem cells proliferate in response to EGF and FGF in the developing mouse telencephalon. *Dev. Biol.* 208:166–188.

Vanderluit, J.L., K.L. Ferguson, V. Nikoletopoulou, M. Parker, V. Ruzhynsky, T. Alexson, S.M. McNamara, D.S. Park, M. Rudnicki, and R.S. Slack. 2004. p107 regulates neural precursor cells in the mammalian brain. *J. Cell Biol.* 166:853–863.

Wallace, V.A., and M.C. Raff. 1999. A role for Sonic hedgehog in axon-to-astrocyte signalling in the rodent optic nerve. *Development.* 126:2901–2909.

Yoshikawa, K. 2000. Cell cycle regulators in neural stem cells and postmitotic neurons. *Neurosci. Res.* 37:1–14.

Yuan, Y., H. Shen, D.S. Franklin, D.T. Scadden, and T. Cheng. 2004. In vivo self-renewing divisions of haematopoietic stem cells are increased in the absence of the early G1-phase inhibitor, p18INK4C. *Nat. Cell Biol.* 6:436–442.