Published in final edited form as:
Dev Neurobiol. 2009 January ; 69(1): 1–21. doi:10.1002/dneu.20680.

Patterns of p57Kip2 Expression in Embryonic Rat Brain Suggest Roles in Progenitor Cell Cycle Exit and Neuronal Differentiation

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Abstract

In developing central nervous system, a variety of mechanisms couple cell cycle exit to differentiation during neurogenesis. The cyclin-dependent kinase (CDK) inhibitor p57Kip2 controls the transition from proliferation to differentiation in many tissues, but roles in developing brain remain uncertain. To characterize possible functions, we defined p57Kip2 protein expression in embryonic day (E) 12.5 to 20.5 rat brains using immunohistochemistry combined with markers of proliferation and differentiation. p57Kip2 was localized primarily in cell nuclei and positive cells formed two distinct patterns including wide dispersion and laminar aggregation that were brain region-specific. From E12.5 to E16.5, p57Kip2 expression was detected mainly in ventricular (VZ) and/or mantle zones of hippocampus, septum, basal ganglia, thalamus, hypothalamus, midbrain and spinal cord. After E18.5, p57Kip2 was detected in select regions undergoing differentiation. p57Kip2 expression was also compared to regional transcription factors, including Ngn2, Nkx2.1 and Pax6. Time course studies performed in diencephalon showed that p57Kip2 immunoreactivity co-localized with BrdU at 8 hr in nuclei exhibiting the wide dispersion pattern, whereas co-localization in the laminar pattern occurred only later. Moreover, p57Kip2 frequently co-localized with neuronal marker, β-III tubulin. Finally, we characterized relationships of p57Kip2 to CDK inhibitor p27Kip1: In proliferative regions, p57Kip2 expression preceded p27Kip1 as cells underwent differentiation, though the proteins co-localized in substantial numbers of cells, suggesting potentially related yet distinct functions of Cip/Kip family members during neurogenesis. Our observations that p57Kip2 exhibits nuclear expression as precursors exit the cell cycle and begin expressing neuronal characteristics suggests that the CDK inhibitor contributes to regulating the transition from proliferation to differentiation during brain development.

Keywords

Cyclin-Dependent Kinase Inhibitor p57Kip2; Embryonic Development/physiology; Nervous System/cytology/*embryology; Brain/embryology; Neuronal Differentiation

Introduction

During development of the central nervous system (CNS), neuronal populations along the rostrocaudal axis are produced from proliferative precursor cells that line the ventricular system. Differences in neuronal populations along the axis presumably reflect expression of

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distinct transcription factors that are thought to define regional cell position and differentiation (DiCicco-Bloom and Sondell, 2005). Despite regional differences, there is tight correlation of proliferative precursor withdrawal from the cell cycle and onset of neuronal differentiation. While proliferative precursors are localized to the ventricular zone (VZ), and to overlying subventricular zone (SVZ) in the forebrain, cells that have exited the cycle and begun differentiation are radially displaced in the more distal mantle zone. Thus the transition from proliferation to differentiation classically correlates with cell migration from the VZ to regions of terminal differentiation.

Withdrawal of mitotic precursors from the cell cycle is under control of two families of mitotic inhibitory proteins that are described as cyclin-dependent kinase (CDK) inhibitors, the Cip/Kip and Ink4 families. The Ink4 (inhibitors of CDK4) family includes p15Ink4b, p16Ink4a, p18Ink4c, p19Ink4d (Elledge and Harper, 1994), proteins that inhibit specifically the activities of CDK4 and CDK6. These proteins contain multiple ankyrin repeats and bind specifically to CDK4 and CDK6 proteins. In developing mouse nervous system, there is differential Ink4 protein expression, with both p18Ink4c and p19Ink4d apparently involved in neurogenesis while p15Ink4b and p16Ink4a are absent (Zindy et al., 1997a; Zindy et al., 1997b; Zindy et al., 1999). p18Ink4c and p19Ink4d are expressed in developing neonatal anterior SVZ (Coskun and Luskin, 2001; Legrier et al., 2001; Luskin and Coskun, 2002) and cerebellum (Watanabe et al., 1998). The expression pattern of p19Ink4d has been associated with progenitor cell cycle exit in the neonatal anterior SVZ and the rostral migratory stream.

The Cip/Kip family of inhibitors is more broadly acting, their actions affecting the activities of cyclin D-, E-, and A-dependent kinases. The family is comprised of p21Cip1, p27Kip1 and p57Kip2. The Cip/Kip proteins bind to both cyclins and CDK subunits, either alone or as molecular complexes. By binding the cyclin E/CDK2 complex, family members prevent the critical transition from G1 into S phase, which establishes commitment to DNA synthesis and cell division (Sherr and Roberts, 1999). Previous studies indicate that family members affect cell cycle regulation in several neural regions including the retina, pituitary gland, and cerebral cortex (Franklin et al., 1998; Carey et al., 2002; Coqueret, 2002; Sumrejkanchanakij et al., 2003). However, recent studies indicate that family members play additional roles in cell differentiation. For example, p21Cip1 regulates cell cycle exit and process outgrowth of retinal precursor cell (Tanaka et al., 2002). p27Kip1 is necessary for cell cycle exit of oligodendrocyte precursors as well as astrocytes (Koguchi et al., 2002; Crockett et al., 2005), and regulates specific stages of neural precursor differentiation in the SVZ (Casaccia-Bonnefil et al., 1997; Doetsch et al., 2002; Bryja et al., 2005; Ishizaki, 2006; Paris et al., 2006) and olfactory epithelium (Legrier et al., 2001). In addition, p27kip1 regulates differentiation and radial migration of cortical projection neurons (Nguyen et al., 2006; Itoh et al., 2007). And in combination, p27Kip1 and p19Ink4d apparently maintain differentiated neurons in a quiescent state in the postmitotic brain (Zindy et al., 1999).

In comparison with p21Cip1 and p27Kip1, p57Kip2 protein has distinct structural features and exhibits different patterns of developmental expression, with prominent levels in embryonic tectum (Lee et al., 1995; Matsuoka et al., 1995; Zhang et al., 1997). p57Kip2 plays an essential and uncompensated role as it is the only member whose deletion produces early postnatal death. In the brain, p57Kip2 is expressed in postmitotic dopamine neurons, influencing their survival and differentiation in the midbrain (Joseph et al., 2003). In the retina, p57Kip2 is involved in cell cycle exit at early times, and later, in differentiation of a subset of amacrine interneurons (Dyer and Cepko, 2000; Dyer and Cepko, 2001). The importance of p57Kip2 in brain neurogenesis has been suggested by its role in mediating the anti-mitogenic effects of PACAP in primary cerebral cortex precursors of the embryonic rat (Suh et al., 2001; Carey et al., 2002). In addition, recent studies using peripheral neural crest-derived neuroblastoma cell lines indicate that bHLH transcription factors target

p57Kip2 to induce cell cycle withdrawal (Rothschild et al., 2006). Although expression of p57Kip2 and other family members in embryonic brain has been reported previously, only mRNA transcripts were examined and analyses were regionally limited (van Lookeren Campagne and Gill, 1998). Further, cell cycle protein levels depend significantly on posttranslational mechanisms, especially proteosomal degradation, leading to poor correlation of CDK inhibitor protein to mRNA levels (Nguyen et al., 2006). Thus the expression and possible roles of p57Kip2 protein in developing CNS has not been fully characterized. Recently we described highly sensitive methods to detect p57Kip2 protein immunoreactivity in the rat embryo (Ye and DiCicco-Bloom, 2005), though relationships to the cell cycle and regions of differentiation remain to be defined. We now report that p57Kip2 protein is expressed primarily in postmitotic cell nuclei that exhibit regionally specific spatial and temporal patterns. p57Kip2 expression is strictly associated with cells recently exiting the cycle and beginning differentiation in the VZ and mantle zone as they migrate to regions of neuronal maturation. Furthermore, p57Kip2 and p27Kip1 appear to be expressed sequentially in subsets of cells, suggesting the two members contribute related though distinct functions during neurogenesis.

Material and Methods

Animals

Time-mated pregnant Sprague Dawley rats were obtained from Hilltop Lab Animals (Philadelphia, PA) and were housed and treated according to approved guidelines of the UMDNJ IACUC and NIH regulations. Embryos from gestational days 10.5 (E10.5) to E20.5 were used.

BrdU injections and tissue processing

Thymidine analog BrdU was used as a proliferation marker to label cells replicating DNA in S phase at the time of administration. Pregnant dams were injected intraperitoneally with BrdU (100mg/kg) and, following deep anesthesia with a mixture of ketamine 30mg/kg and xylazine 3mg/kg, were sacrificed at the indicated time points to remove embryos. For embryos of E14.5 or younger, the entire head was transected and fixed directly overnight at 4°C in cold 4% paraformaldehyde in 0.1M PBS, pH7.4, while for E15.5 or older, the brain was first dissected from the skull and then fixed using the same protocol. After a 3 hr rinse with cold PBS, tissues were cryoprotected with 30% sucrose at 4°C for 2–3 days until they sank. Tissues were embedded in O.C.T, frozen on dry ice, cut in the coronal plane on a cryostat at 12μm, and mounted on Superfrost plus slides (VWR, Bridgeport, NJ). Sections were dried at least 2 hr at room temperature (RT), and then stored at −20°C until use.

Immunohistochemical procedures

For immunohistochemical staining of p57Kip2, at least two embryos from different pregnancies were used at E10.5, E12.5, E14.5, E15.5, E16.5, E18.5 and E20.5. Sections were pretreated with DNase I, exposed to goat anti-p57Kip2 primary antibody, then horse anti-goat secondary, followed by Vector ABC development and enhancement with biotinyl tyramide reagent, using methods described previously (Ye et al., 2007). Prior to DNase I treatment, tissue sections were rinsed 3 times 15 min in PBS (pH 7.2), incubated in 3% H_2O_2 (diluted in methanol) for 30min, rinsed again 3 times 15 min in PBS, then incubated in PBS containing 0.3% Triton X-100 (PBS-T) for 10 min. For DNase I treatment, sections were incubated with DNase I buffer for 5 min followed by DNase I (500U/ml) for 10 min. After 4 washes in de-ionized water over 40 min, tissues were blocked for 1 hr in blocking buffer containing 10% normal horse serum (NHS), 3% bovine serum albumin (BSA, Sigma) in 0.3% PBS-T. Sections were incubated with goat anti-p57Kip2 antibody (E-17, 1:200; Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 0.2% PBS-T containing 5% NHS and

1% BSA, for 3 days at 4°C. After PBS rinses, sections were incubated for 1 hr at RT with biotinylated horse anti-goat antibody (1:800; Vector Lab, Burlingame, CA) prepared in 0.2% PBS-T containing 1% NHS and 1% BSA, then rinsed with PBS, and incubated with *Elite* ABC kit (Vector Lab) for 60 min. After rinses, signal was further amplified by incubating sections for 10 min with biotinyl tyramide solution according to the manufacturer's instructions (1:100; NEL700, PerkinElmer, Waltham, MA). Following rinses, antigen was revealed using fluorescein-avidin D (1:2000; Vector Lab), diluted in PBS containing 1% BSA, for 1 hr.

For double immunofluorescent labeling, p57Kip2 staining was performed first as above, then, after blocking with 5% BSA in 0.3% PBS-T at RT for 30 min, sections were incubated with different primary antibodies overnight at 4°C. Other primaries were mouse anti-BrdU (1:75; BD Biosciences, San Diego, CA), mouse anti-β-III tubulin (Tuj1, 1:1000; Covance, Emeryville, CA), mouse anti-MAP2 (1:1000; Sigma, St. Louis, MO), mouse anti-p27Kip1 (1:1000; BD Biosciences), rabbit anti-Neurogenin 2 (1:200; gift from Yongchao Ma, Harvard Univ.), rabbit anti-Nkx2.1 (1:500; gift from Michael Matise, Robert Wood Johnson Medical School), rabbit anti-Ki67 (1:500; Novocastra) and rabbit anti-Pax6 (1:1000; Chemicon). After four 10 min washings in PBS, slides were incubated for 1hr at RT with the appropriate secondary antibodies conjugated to Alexa Fluor 488 or 594 (1:1000; Molecular Probes). Following PBS rinses, sections were mounted using ProLong Gold antifade reagent (Molecular Probes) for images analysis.

For p57Kip2/BrdU/Tuj1 triple labeling, sections were sequentially stained for p57Kip2, then rat anti-BrdU (1:75; BD Biosciences), and finally mouse anti-Tuj1. Rat anti-BrdU antibody was revealed using chicken anti-rat Alexa Fluor 647 and mouse anti-Tuj1 antibody with rabbit anti-mouse Alexa Fluor 594.

Microscope and image analysis

Images were acquired using fluorescence microscopy on a Zeiss Axiovert 200M. The ApoTome program was used to acquire Z stacks of 0.45 μm on 40x or 63x magnification images. Acquired images were analyzed using AxioVision software. Triple labeling images were acquired using a laser scanning confocal microscope (Zeiss).

Quantification of immunolabeled cells on E14.5 was performed on two or more sections from each of two or more embryos in each region, examining from 350–500 cells per section. When assessing the BrdU labeling index, positive cells were compared to total nuclei defined by DAPI staining. The BrdU labeled region was used to define the proliferative zone in various brain areas and regions more laterally placed were termed as non-proliferative zone. These regions were then used to characterize the proportion of CKI markers expressed in each. For assessing double labeling of p57Kip2 and p27Kip1, p57Kip2-expressing cells in both the proliferative and non-proliferative zones were enumerated and then the percent that also expressed p27Kip1 was determined.

Results

Expression of p57Kip2 protein in the ventricular (VZ), subventricular (SVZ) and mantle zones of the embryonic rat brain

Defining the patterns of p57Kip2 expression in the embryonic brain may provide clues regarding its roles during CNS development. As a cell cycle regulator, it was critical to characterize p57Kip2 expression in relation to mitotic S phase. To accomplish this goal, we recently designed a new method that enhances detection of p57Kip2 immunoreactivity in nuclei of embryonic brain tissues, increasing both signal intensity and numbers of positive cells. Furthermore, since we found that p57Kip2 protein was especially labile to HCl

exposure commonly used for BrdU immunolabeling, or citrate antigen retrieval, the alternative use of an optimized DNase I pretreatment protocol allowed successful study of developmental expression (Ye et al., 2007). First, we defined p57Kip2 protein expression during prenatal development from E12.5 to E20.5, an age range that targets the period of major neurogenesis in the forebrain. Our observations indicate that p57Kip2 expression is detectable at almost all levels of the CNS during E12.5 to E16.5 from forebrain to spinal cord. Furthermore, p57Kip2 expression exhibited both region-specific and time-dependent patterns throughout the neural axis (Table 1). During this period, the majority of p57Kip2 immunoreactivity we detected was confined to the nucleus in cells localized to the VZ, SVZ and emerging mantle zones (Fig. 1), where precursors are undergoing cell cycle progression or cycle withdrawal and initiation of differentiation. High cellular densities and signal intensities of p57Kip2 immunoreactivity were detected in the septal area, basal ganglia, preoptic area, epithalamus, thalamus, hypothalamus, midbrain and spinal cord (Fig.1; Table 1). In other forebrain regions, the density of p57Kip2 positive cells was significantly lower at the same embryonic stages (E12.5 to 16.5; Table 1).

Cells exhibiting p57Kip2 immunoreactivity appeared generally in one of two distinct expression patterns. In the first pattern, cells expressing p57Kip2 demonstrated an intermediate tissue density and relatively wide dispersion in regions surrounding the ventricles, such as observed in the basal ganglia, septum and hippocampus (Fig. 1A–D). Alternatively, p57Kip2-expressing cells formed a more dense laminar aggregation at the basal extent of the proliferative zone, as seen in the thalamus, midbrain, preoptic area and spinal cord (Fig. 1E–H). Significantly, these expression patterns were region-specific, as described below, and did not exhibit change over the course of development.

p57Kip2 has proposed roles in cell cycle exit and the onset of differentiation during neurogenesis. To begin defining relationships to the cell cycle, we examined p57Kip2 expression in combination with incorporation of thymidine analog, bromodeoxyuridine (BrdU), which labels cells in S phase undergoing DNA synthesis. Embryos were processed for double immunohistochemistry 1–2 hr after BrdU was administered to pregnant dams. We found that p57Kip2 did not exhibit a simple relationship to BrdU labeled proliferative precursors. Rather, in different CNS regions, p57Kip2 immunoreactivity was detected in subsets of cells (Fig. 2A, B) and positive cell density varied as well (Table 1). For example at E12.5, p57Kip2 immunoreactivity was present throughout the dorsoventral and mediolateral extents of the CNS (Fig. 2A). A variety of spatial patterns of p57Kip2 expression are exemplified in a coronal section through the diencephalon at E14.5. Differences in the expression patterns are striking when comparing the thalamus to the hypothalamus and epithalamus surrounding the third ventricle (Fig. 2B, Table 1). Overall, p57Kip2 immunoreactivity was present in the nuclei of progenitors with an increased density of positive cells at the basal margins of the VZ (Fig. 2E, F, I, J). With further development, however, p57Kip2 expression in the ventricular regions was significantly down-regulated by E18.5 and 20.5 (Fig. 2G, K; Table 1).

While both p57Kip2-expressing and BrdU positive cells were distributed in the same or closely adjacent regions, double labeled cells were rarely observed using the Zeiss Axiovert 200M ApoTome microscope. For further analysis, we employed Zeiss confocal imaging and found no p57Kip2/BrdU double-labeled cells in the thalamus or hypothalamus at 1–2 hr (Fig. 2C, D), indicating that p57Kip2 is not expressed in S, G2 or M phases and implying that p57Kip2 is expressed by cells in G1 phase of the cell cycle. The studies also revealed that p57Kip2 positive nuclei were smaller than those labeled by BrdU (insets in Fig. 2C, D), though a quantitative analysis was not performed.

By analyzing the spatial distribution of cells exhibiting p57Kip2 and BrdU immunoreactivities in the thalamus and hypothalamus at E14.5, we further characterized the two patterns of p57Kip2 expression, described above as wide dispersion and laminar aggregation. In the wide dispersion pattern exemplified by the hypothalamus (Fig. 2B), p57Kip2 expressing cells were widely dispersed among the BrdU labeled cells, forming a mosaic pattern (Fig. 2I, J). The density of p57Kip2 positive cells increased with the distance from the apical surface that lines the ventricles and appeared highest at the basal margins of the VZ or just outside the zone of BrdU labeled progenitors. Beyond this margin of p57Kip2 expression, signal rapidly diminished in the neuronal differentiation layer at E14.5 and thereafter. We quantified the percentage of cells in the proliferative and non-proliferative zones that exhibited either BrdU or p57Kip2 immunoreactivity, expressed as a ratio to total cells stained with DAPI (Table 2). In the proliferative region of the hypothalamus, approximately 15% of cells were BrdU labeled whereas a separate 52% expressed p57Kip2. In the non-proliferative zone, ~1% and 2.4% were labeled with BrdU and p57Kip2, respectively. This wide dispersion, mosaic pattern was also found in the proliferative regions of the basal ganglia, septum, epithalamus and hippocampus (Table 1, Fig. 1A–D, Fig. 3A, D, E). The second, dense laminar aggregate pattern appeared most prominently in the thalamus at E14.5 (Fig. 2B), in which p57Kip2 positive cells were localized as a dense aggregate lateral to the BrdU positive zone (Fig. 2E, F). Earlier, at E12.5, there were fewer p57Kip2 positive cells in the thalamic neuroepithelium, some of which were within the VZ (Fig. 2E). With further development, p57Kip2-expressing cells increased markedly in number and were concentrated almost entirely outside the proliferative region (Fig. 2F). In the thalamic proliferative zone, 50% of cells labeled with BrdU and only 3.5% with p57Kip2, whereas 63% of the cells outside the VZ expressed p57Kip2, a striking difference when compared to the hypothalamus (Table 2). This laminar aggregate pattern was also observed in the preoptic area, midbrain and spinal cord (Fig. 1E–H, Fig. 3B, C, F). By comparing the wide dispersion and laminar aggregation patterns in these several tissues we also asked whether they may correlate with regions possessing an SVZ, which originates from the VZ and serves as a secondary proliferative zone (Brazel et al., 2003;DiCicco-Bloom and Sondell, 2005). However, this did not seem to be the case: the wide dispersion pattern was observed in basal ganglia, septum, epithalamus which possess an SVZ as well as in hypothalamus and hippocampus which lack this secondary zone. Similarly, the laminar aggregation pattern was observed in the thalamus that has an SVZ as well as the midbrain and spinal cord, where the SVZ is absent.

In addition to proliferation zones, p57Kip2 was also expressed in certain areas undergoing neuronal differentiation (Table 1). p57Kip2 positive cells were prominent in the neural differentiation field of the thalamus at E18.5 and later (Fig. 2G, H). In contrast, p57Kip2 was not expressed in the differentiating field of the directly adjacent hypothalamus at later ages (Fig. 2K, L). The sequential expression of p57Kip2 in progenitors followed by later expression during differentiation was also found in the septal area as well as preoptic area, and therefore did not correlate specifically with the two early patterns of p57Kip2 expression (Fig. 1). In the cerebral cortex from E12.5 to E16.6, we detected low levels of nuclear p57Kip2 in a subset of cells in both VZ/SVZ as well as the cortical plate (Fig. 2A; Table 1), though this will be the subject of a separate manuscript. Overall, these observations indicate that p57Kip2 is expressed in regionally restricted spatial-temporal patterns during prenatal neurogenesis, localized to the VZ, SVZ and mantle zones at early stages and subsequently in select fields of neuronal differentiation.

Relationship of p57Kip2 expression to markers of developmental stages and specific regional compartments

To further clarify p57Kip2 expression patterns, we compared them to that of several makers of cell development and lineage at E14.5, focusing on the hypothalamus which exhibits the wide dispersion pattern, only commenting on the thalamus when observations differ (Fig. 4). For an overview of the proliferative region, we examined p57Kip2 and nestin, a well-characterized marker of neural precursors expressed in cell bodies as well as processes of the radial glial cells (Noctor et al., 2001;Noctor et al., 2004). The majority of p57Kip2-expressing cells fell within the nestin domain (Fig. 4A). However, as a cytoplasmic filament, nestin immunoreactivity can be difficult to co-localize with nuclear markers, such as p57Kip2. To more specifically label precursors, we used two different nuclear makers of cells engaged in proliferation, PCNA and Ki67(Scholzen and Gerdes, 2000;Kee et al., 2002;Muskhelishvili et al., 2003). By comparing neighboring sections, we found that PCNA extended over a wider apicobasal extent than Ki67 (Fig. 4B, C). p57Kip2 immunoreactive cells were localized to regions where cells were expressing either PCNA or Ki67. In the case of PCNA, the overwhelming majority of p57Kip2-expressing cells also expressed PCNA, with 90±7% of cells exhibiting double labeling in the hypothalamus and 92±3% in the thalamus, suggesting that the cells were either still engaged in the cell cycle, such as G1 phase, or alternatively, recently withdrawn from the cycle. This model may be consistent with the apparent inverse correlation in signal intensities of PCNA and p57Kip2 we observed but did not quantify. In comparison, for Ki67 expression which is known to be more tightly correlated with cell cycle stages, only 50±2% of p57Kip2-expressing cells exhibited Ki67 in the hypothalamus and 14±5% of cells exhibited double labeling in the thalamus.

We also examined relationships of p57Kip2 expression in the diencephalon to several transcription factors whose domains of expression serve to pattern cell position and identities in the CNS, including neurogenin 2 (Ngn2), Nkx 2.1 and Pax6 (Tuttle et al., 1999; Bishop et al., 2000; Kawaguchi et al., 2004; Miyata et al., 2004). Overall, p57Kip2 immunoreactivity did not co-localize absolutely with any of these factors. In the hypothalamus, Nkx2.1 was expressed in a wide domain (Fig. 4D), similar to nestin (Fig. 4A) and p57Kip2 was expressed in a more narrow, periventricular region (Fig. 4F). In the upper part of the hypothalamus, Ngn2 exhibited a narrow band of immunoreactivity adjacent to the ventricle, consistent with the VZ, and p57Kip2 immunoreactive cells exhibited some co-localization as well as isolated expression in more laterally placed cells (Fig. 4E). Double labeling was also apparent though less common for Ngn2 and p57Kip2 in the thalamus (Fig. 4F). Finally, unlike these markers of proliferative precursors, Pax6 was expressed more laterally in regions of neural differentiation, and exhibited no double labeling with p57Kip2, whose expression localized medial to Pax6 (Fig. 4G). These observations suggest that these markers of developmental compartments did not define the cells that express p57Kip2, which protein seemed to straddle the proliferative and differentiative regions in these brain structures.

Temporal relationship of p57Kip2 expression to S phase entry in proliferative regions

To further define the relationship of p57Kip2 expression to cell cycle progression and withdrawal, we performed p57Kip2/BrdU double immunostaining in E14.5 embryos at different time points after BrdU injection including 1, 8 and 24 hr. Representative images from the diencephalon are shown for illustration (Fig. 5). Analyses were performed in the hypothalamus, a structure exhibiting the wide dispersion pattern of p57Kip2 expression, and the thalamus, in which p57Kip2-expressing cells form a dense laminar aggregate. At 1 hr, BrdU labeled precursors in both structures were localized to more basal regions of the VZ as expected for cells engaged in S phase (Fig. 5,1 hr). The cells expressing p57Kip2 formed a

separate and distinct subpopulation of cells and exhibited no double labeling. At 8 hr, many BrdU labeled cells were closer to the ventricle, having migrated to the apical region from their initial basal location. In the hypothalamus, a small subset of BrdU labeled cells expressed p57Kip2, while this co-localization was absent or rarely observed in the thalamus. This may suggest that p57Kip2 plays a different role in each structure, or alternatively, that the cell cycle times or modes of division differ between the two. By 24 hr (Fig. 5), the number of cells double labeled for BrdU and p57Kip2 increased in the hypothalamus, and double labeling was now observed in the thalamus. Interestingly, in the epithalamus, another structure that exhibits the wide dispersion, mosaic pattern, p57Kip2/BrdU double labeling first appeared at 8 hr and increased further by 24 hr, recapitulating the pattern defined in the hypothalamus. This suggests that regions exhibiting the same p57Kip2 expression patterns possess common cell cycle kinetics. In all these structures, p57Kip2-expressing cells at 24 hr appear as a band that sits between BrdU positive cells remaining in the proliferative region and BrdU labeled cells on their way to zones of differentiation. It is unclear whether p57Kip2 is expressed at a precise but brief moment in all precursors during development, or alternatively, in only a distinct cell subset.

Expression of p57Kip2 occurs as precursors initiate neuronal differentiation

As the foregoing observations suggested that p57Kip2 expression correlates with cell cycle withdrawal, we characterized its relationships to differentiation using double and triple immunolabeling with p57Kip2, BrdU and neuron specific cytoskeletal marker β-III tubulin (TuJ1). Overall, p57Kip2 expression was localized to the boundary zone between regions of proliferation and differentiation at E12.5–14.5. In the telencephalon, diencephalon and spinal cord, p57Kip2 immunoreactivity was observed at the transitional region between the VZ and cells expressing TuJ1 (Fig. 6). Significantly, a high proportion of cells were double labeled for nuclear p57Kip2 and TuJ1, including E14.5 epithalamus (Fig. 6C), thalamus (Fig. 7A–C) and hypothalamus, ranging from 60% to 85% of cells assessed using confocal image analysis. In the thalamus, we found 70.8±7.5% (mean±SD) of cells were double labeled. In the basal hypothalamus, the proportion was similar at 81.4±6.8%. However, in some structures where p57Kip2-expressing cells localized more medially, in the apical VZ, this proportion appeared to be lower, though this was not quantified. Furthermore, by adding a BrdU pulse analysis at 2 and 8 hr, we assessed the apparent timing of cell cycle exit and differentiation. At 2 hr, p57Kip2-expressing cells surrounding the third ventricle co-labeled with TuJ1 but did not exhibit BrdU staining (Fig. 7A–C). In contrast, by 8 hr, a subset of cells labeled with BrdU had now migrated towards regions of differentiation and also expressed p57Kip2 as well as TuJ1 (Fig. 7D–G). These relationships were present in the E14.5 hypothalamus, septal area and basal ganglia. These data indicate that as cells exit the cycle, p57Kip2 is expressed in parallel with the differentiation marker, β-III tubulin, and suggest that increased expression of p57Kip2 is associated with neuronal differentiation. We also noted that some cells at 8 hr exhibited p57Kip2 and BrdU but failed to express TuJ1, which may potentially reflect precursors not destined to become neurons or simply the technical limitations of associating cytoplasmic filaments with nucleus-localized signals.

Potential cooperative roles of p57Kip2 and p27Kip1 in E14.5 developing forebrain

As p27Kip1 and p57Kip2 are members of the same CKI family and exhibit significant sequence similarity (Lee et al., 1995), they may act cooperatively to sequentially regulate the transition from proliferation to differentiation in individual cells, or alternatively, function in parallel or redundant fashion. We examined the spatial expression patterns of p27Kip1 and p57Kip2 proteins in E14.5 brain by using combined immunostaining, and compared patterns to BrdU and TuJ1 in the same or adjacent sections. Cells exhibiting p27Kip1 and p57Kip2 immunoreactivity were distributed in distinct and primarily adjacent embryonic brain regions. p57Kip2 positive cells were primarily situated in proliferative

zones, with signal diminishing rapidly as one approaches regions of differentiating neurons (Fig. 8A). In contrast, p27Kip1 immunoreactivity was expressed in a uniform fashion lateral to the p57Kip2 positive zone, in regions exhibiting neuronal marker TuJ1 in the diencephalon. Expression of p27Kip1 was rare and of low intensity in the VZ when present. To further define relationships, confocal analysis was employed on sections of the epithalamus, where double staining was most robust. Cells exhibiting p57Kip2 nuclear signal appeared to be located closer to the ventricles, within the VZ/SVZ, whereas the majority of p27Kip1 cells were displaced laterally, in areas of differentiation that expressed TuJ1 (Fig. 8B). However, a subset of nuclei at this transitional zone exhibited double immunolabeling for p27Kip1 and p57Kip2 (Fig. 8B). Similarly, in E14.5 hypothalamus, p57Kip2 expression was localized medially as a mosaic with BrdU labeled precursors, and p27Kip1 and TuJ1 were localized more laterally with evidence of CKI co-localization at the boundary (Fig. 8C).

We also characterized the distribution of p57Kip2-expressing cells in the diencephalon in both the proliferative and non-proliferative regions, defined by BrdU labeling in adjacent sections. In the thalamus that exhibits a laminar aggregate pattern, less than 5% of p57Kip2-expressing cells were localized to the proliferative region and greater than 95% were in the non-proliferative zone (Table 3). In contrast, two structures exhibiting a wide dispersion, mosaic pattern, the hypothalamus and epithalamus, localized 92% and 74% of p57Kip2-expressing cells respectively in the proliferative zones. With regard to p27Kip1, however, the distribution of this signal in the same locations was between 20 and 30% in the proliferative regions, suggesting little correlation with p57Kip2 (Table 3). Finally, the rare p57Kip2 positive cells in the thalamic proliferative zone infrequently co-localized p27Kip1, whereas double labeling was far more common in mosaic proliferative zones, with 34% in the epithalamus and 68% in the hypothalamus. While underlying mechanisms remain to be defined, these observations suggest that cells undergo sequential expression of CKI family members as they develop, with a transitional period of overlapping expression that may be related to regional cell cycle parameters. Thus, transient p57Kip2 signal may be replaced by sustained p27Kip1 expression as neuronal progenitors transition from proliferation to differentiation during developmental neurogenesis.

Discussion

In the developing nervous system, cell cycle exit is tightly coupled to neuronal differentiation. While members of the Cip/Kip family of CDK inhibitors may serve to elicit cell cycle withdrawal, the roles of p57Kip2 in developing brain are not fully explored. Our observations indicate that p57Kip2 protein is expressed throughout the neural axis during neurogenesis and exhibits both region-specific and time-dependent patterns. From E12.5 to E16.5, p57Kip2 immunoreactivity was present in the VZ, SVZ and/or mantle zone, localized primarily to cell nuclei. Cells exhibiting p57Kip2 generally appeared in one of two distinct patterns of expression: p57Kip2-expressing cells were either widely dispersed among the mitotic, BrdU labeled cells in the VZ, forming a mosaic, or alternatively, p57Kip2 positive nuclei were localized as a dense aggregate outside the proliferative zone. The distinct patterns of expression raise the possibility that p57Kip2 serves different functions in specific brain regions, or reflect distinct regional cell cycle kinetics during neurogenesis. At E18.5 and later, p57Kip2 protein was expressed in nuclei of a distinct subset of differentiating fields. Time lapse studies indicated that p57Kip2 protein was expressed at about 8 hours after cells were in S phase in regions exhibiting a dispersed, mosaic pattern, but only later for structures exhibiting the dense laminar pattern. Moreover, p57Kip2 expression occurred in parallel with neuronal differentiation marker, β-III tubulin, placing these cells at the boundary between cell proliferation and neuronal differentiation. Furthermore, in relation to another family member, p27Kip1, the proteins were distributed in distinct and primarily

adjacent tissue domains, with p57Kip2-expressing cells located closer to the ventricles, whereas the majority of p27Kip1 cells were displaced laterally, in areas of differentiation. Nonetheless, substantial CKI co-labeling was observed. These observations suggest that p57Kip2 is expressed as cells exit the cycle and begin differentiation and that, as cells migrate radially, transient p57Kip2 signal is replaced by sustained expression of p27Kip1.

The detection of high levels of p57Kip2 nuclear immunoreactivity in embryonic brain regions including the basal ganglia, septum, amygdala, hippocampus, preoptic area, epithalamus, thalamus, hypothalamus, midbrain and spinal cord from E12.5 to E16.5 suggests that the inhibitor provides a general signal during the transition from proliferation to differentiation in the nervous system. This is consistent with its critical anti-mitogenic role throughout the embryo, where it negatively regulates the cell cycle and promotes cell differentiation (Lee et al., 1995; Matsuoka et al., 1995). Deletion of p57Kip2 in mice is fatal, and causes hyper-proliferation and failed differentiation in the many organs in which it is specifically expressed (Lee et al., 1995; Zhang et al., 1997; Zhang et al., 1999; Takahashi and Nakayama, 2000). p57Kip2 mRNA was initially detected in embryonic brain in the tectum, and more recently, in the VZ/SVZ of the neocortex, ganglionic eminences, and cortical plate in the latter half of neurogenesis, E16 to E18. p57Kip2 mRNA is also up-regulated later at E20 in fully differentiated neurons (Lee et al., 1995; Matsuoka et al., 1995; van Lookeren Campagne and Gill, 1998; Nagahama et al., 2001; Itoh et al., 2007). In contrast to the mRNA transcript, expression of p57Kip2 protein in most embryonic brain regions has not been reported. It is especially important to define CKI protein expression because it is frequently controlled by post-translational mechanisms, including protein degradation through the proteasome, sequestration, and nuclear/cytoplasmic translocation, leading to discrepancies in mRNA and protein levels (Urano et al., 1999; Nishimori et al., 2001; Fujita et al., 2003; Leibovitch et al., 2003; Quintanilla-Martinez et al., 2003; Nguyen et al., 2006; Surjit and Lal, 2007). Our observation that p57Kip2 protein is expressed throughout the neural axis at E12.5 suggests it may contribute to precursor cell symmetric divisions that generate additional stem cells. The subsequent spatial and temporal patterns of p57Kip2 expression in diverse brain structures suggest potential roles in controlling cell division and neural differentiation that underlie regional neurogenesis (DiCicco-Bloom and Sondell, 2005; Gotz and Huttner, 2005).

Our studies identified two distinct patterns of cells expressing p57Kip2 protein, forming a mosaic with BrdU labeled cells within the proliferative region (in hypothalamus, epithalamus), or alternatively, forming a dense layer just lateral to the proliferative zone (thalamus). Significantly, the patterns were region specific, and did not change over the course of development. These patterns may reflect differing p57Kip2 functions, such as eliciting cell cycle exit of precursors when in mosaic form as they traverse from apical to basal regions, whereas the laminar aggregate outside the proliferative zone may indicate a role in the onset of migration and/or neural differentiation. While we do not fully understand its functions, ongoing studies examining the p57Kip2 knock out mouse and over-expression techniques (Tury et al., 2006) may help address these issues. Alternatively, these expression patterns may not reflect differences in protein function, but rather specific characteristics of the neural precursors themselves in region. For example, different regions may possess distinct cell cycle times (see below), may undergo different temporal profiles of symmetric versus asymmetric cell division or may display distinct neurogenetic spatiotemporal gradients. Moreover, in the diencephalon, there is differential expression of retinoid X receptors, with beta receptors in thalamus and gamma in hypothalamus (Moreno et al., 2004), as well as thyroid receptors (Vercelli et al., 2007), raising the possibility that different cellular mechanisms control precursor proliferation that is also reflected in expression of specific cell cycle regulators.

Several lines of evidence indicate a role for p57Kip2 in regulating cell cycle exit of neural progenitors. Levels of p57Kip2 protein and its association with the cyclin E/CDK2 kinase complex were increased when PACAP treatment inhibited DNA synthesis of cerebral cortical precursors, producing reductions in CDK kinase activity and G1 to S phase transition (Suh et al., 2001; Carey et al., 2002). Further, genetic over-expression of p57Kip2 led to cell cycle exit in retinal precursors (Ohnuma et al., 1999) and G1 blockade in human astrocytoma cell lines (Tsugu et al., 2000). In mouse neuroblastoma cells, p57Kip2 was identified as a functional target recruited by bHLH transcription factors to induce cell cycle arrest (Rothschild et al., 2006). However, little evidence links p57Kip2 expression to cell cycle exit in developing brain. In our current studies, 1–2 hr after injection, BrdU labeled cells were localized to the basal VZ where cells are engaged in DNA synthesis (Noctor et al., 2004; Gotz and Huttner, 2005) and there was no co-localization with p57Kip2. However, by 8 hr, cells have had time to complete S, G2 and M phases, and are now presumably engaged in G1 or G0, as they begin moving away from the ventricle. It was at 8 hr that BrdU co-localized with p57Kip2, suggesting that the inhibitor was first expressed after completion of mitosis during G1 stage, when cells are deciding whether to re-enter the cell cycle or withdraw from proliferation (Noctor et al., 2004; Gotz and Huttner, 2005). This time course was observed in regions exhibiting the wide dispersion, mosaic pattern of p57Kip2 and BrdU expression, including the hypothalamus and epithalamus. The mosaic pattern may reflect a mixture of precursor cells including 1) the p57Kip2-expressing cells that are exiting the cycle and will migrate out of the VZ toward differentiation fields, and 2) the BrdU labeled cells that will re-enter the cell cycle as neural stem cells. Such a model may also be supported by our localization studies of Ki67: Approximately 50% of p57Kip2-expressing cells co-localized Ki67, potentially reflecting cells that completed M phase and have just entered G1, a group of cells in which p57Kip2 will induce cell cycle withdrawal. In contrast, the p57Kip2+/Ki67- cells in the proliferative region would have already exited the cycle and would be engaged in migration and differentiation. In contrast to the mosaic pattern, in regions where p57Kip2-expressing cells form a dense laminar aggregate, such as the thalamus, BrdU co-labeling rarely occurred until after 8 hr, and far fewer cells co-expressed Ki67. This may suggest that p57Kip2 is expressed at later times following the decision to exit the cycle, potentially regulated by other mechanisms. More broadly, these region-specific patterns of expression suggest that p57Kip2 may play distinct roles in different cell populations, or that progenitors possess different cell cycle times or modes of division. Regardless of region, by 24 hr, we observed p57Kip2 expression in a subset of BrdU labeled cells lying between the proliferative zone and the mantle zone of differentiating neurons. These results suggest that p57Kip2 activity serves to elicit cell cycle withdrawal prior to neuronal differentiation. This conclusion is supported by recent studies in spinal cord, in which p57Kip2 expression occurred 12 hr after S entry (Gui et al., 2007).

In addition to blocking cell cycle re-entry, p57Kip2 may also serve to couple mitotic withdrawal with neuronal differentiation, the regulation of which is a central issue in neural development (Bally-Cuif and Hammerschmidt, 2003). Major neurogenesis in developing rat brain occurs from E12.5 to E16.5 (Gotz and Huttner, 2005), the period during which we detected high p57Kip2 expression in proliferative regions. Significantly, the majority of cells expressing p57Kip2 also co-localized βIII-tubulin, suggesting that this CKI identifies the earliest stages of neuronal differentiation. Furthermore, at 8 hr when we first detected co-localization of BrdU with p57Kip2 in the hypothalamus, we also observed p57Kip2 co-expression with βIII-tubulin in many cells, with a subset expressing all three markers. These results support the proposal that p57Kip2 activity is involved with the onset of neuronal differentiation following cell cycle exit, potentially serving as a molecular coupling mechanism. However, a clearer picture may emerge by performing a time course using triple labeling for p57Kip2, BrdU and TuJ1, for which methods are being developed.

A number of studies have explored the possible roles of Cip/Kip family inhibitors in cell differentiation and fate determination. In Xenopus, p27Xic1, an analogue of p27Kip1, was found to induce Muller glia cells from retinoblasts (Ohnuma et al., 1999). In mammals, p27Kip1 has been shown to regulate neuronal differentiation, migration, and proliferation in developing brain (Casaccia-Bonnefil et al., 1997; Durand et al., 1997; Crockett et al., 2005; Itoh et al., 2007). More recently, p27Kip1 was found to promote neuronal differentiation by stabilizing bHLH transcription factor Neurogenin 2 protein in mouse cerebral cortex (Nguyen et al., 2006). A similar stabilizing interaction of p57Kip2 with bHLH transcription factor MyoD was found in muscle development (Reynaud et al., 1999; Reynaud et al., 2000). Further, in p57Kip2 knockout mice, there were numerous tissues exhibiting problems in cell differentiation or fate determination (Zhang et al., 1997). In addition to potential direct interactions, Cip/Kip inhibitors may also contribute to differentiation control by inhibiting cycle-dependant kinases required for this process, such as cyclin E/CDK 2 (Reynaud et al., 1999) and cycle D/CDK4 (Ratineau et al., 2002; Berger et al., 2005). Finally, recent evidence suggests that both p57Kip2 and p27Kip1 play additional roles in regulating neural precursor migration in developing brain (Nguyen et al., 2006; Itoh et al., 2007). In aggregate, these studies support the proposal that Cip/Kip family members promote cell cycle exit, and contribute directly or indirectly to neuronal differentiation, migration and cell fate determination during embryonic development. Our study suggests that these effects may occur at all levels of the developing nervous system.

The fact that p57Kip2 and p27Kip1 possess similar CDK kinase inhibitory activity and share partial structural similarity (Sherr and Roberts, 1995) raises the question of relationships during brain development. Our analysis suggests that p57Kip2 and p27Kip1 expression are precisely coordinated with cell cycle exit and neuronal differentiation. In studies of mutant mice several years ago, p57Kip2 and p27Kip1 were shown to function together in partially redundant fashion in controlling cell cycle exit and differentiation in lens and placenta (Zhang et al., 1998). Further, in the retina, p27Kip1 and p57Kip2 were expressed in different subpopulations of progenitors suggesting they subserved distinct cell types heterogeneously in the timing of cell cycle exit (Dyer and Cepko, 2001). In our current studies, p57Kip2 and p27Kip1 were expressed primarily in different subsets of cells, in adjacent regions. p57Kip2 was expressed in progenitors apparently exiting the cell cycle in proliferative regions. In contrast, p27Kip1 was found mainly in the differentiating neuronal layer where there was intense and sustained expression of β-III tubulin, suggesting p27Kip1 played roles in maintaining cell cycle exit, or differentiation and migration. Significantly, a substantial though variable proportion of p57Kip2-expressing cells at the boundary of the proliferative and differentiative zones also co-expressed p27Kip1. This co-localization suggests there is a coordinated transition within single cells from one to the other Cip/Kip member as cells exit the cycle and initiate differentiation. However, further studies in individual cell types are warranted because the distributions of cells expressing p57Kip2 and p27Kip1 in different brain structures reveal no consistent relationships. At the mechanistic level, it is possible that down-regulation of p57Kip2 in cells may induce p27Kip1 expression. Indeed, recent evidence suggests coordinate CKI regulation: in mouse brain, knockdown of p27Kip1 mRNA led to increased p57Kip2 expression, and alterations in neuronal migration (Itoh et al., 2007). In sum, our studies indicate that p57Kip2 and p27Kip1 expression are under precise regulation during neurogenesis, suggesting that they function in coordinate fashion to control the timing of cell cycle exit and coupling to neuronal differentiation. Whether the two proteins are sequentially expressed in some cells but play separate and distinct functions in others in region-specific fashion remains to be determined. A very specific role of p57Kip2 in survival and differentiation of substantia nigra dopamine (but not other catecholamine) neurons has been demonstrated previously (Joseph et al., 2003), supporting region-specific functions.

Overall, our studies indicate that p57Kip2 exhibits very early and extensive expression in neural precursors as they exit the cell cycle and begin differentiation across the entire neural axis during development. p57Kip2 expression is detected at about 8 hr after a cell has been in S phase, and increases in parallel with differentiation marker, β-III tubulin. While p57Kip2-expressing cells may form a widely dispersed or laminar aggregate pattern, a feature that is region-specific, the protein is expressed transiently, suggesting a temporally discrete role in all cells, or alternatively, a specific role in a subset of precursors. The expression of p57Kip2 in the embryonic brain is precisely regulated with the timing of cell cycle exit of neuronal progenitors and may serve to link this stage with neuronal differentiation (Fig. 9). Finally, p57Kip2 expression primarily precedes p27Kip1, though variable cellular co-expression is observed, suggesting that their expression and function are coordinated to control cell proliferation, migration and neuronal differentiation.

Acknowledgments

Support from National Institute of Neurological Disorders and Stroke grant RO1 NS32401.

References

Bally-Cuif L, Hammerschmidt M. Induction and patterning of neuronal development, and its connection to cell cycle control. Curr Opin Neurobiol. 2003; 13:16–25. [PubMed: 12593978]

Berger C, Pallavi SK, Prasad M, Shashidhara LS, Technau GM. A critical role for cyclin E in cell fate determination in the central nervous system of Drosophila melanogaster. Nat Cell Biol. 2005; 7:56–62. [PubMed: 15580266]

Bishop KM, Goudreau G, O'Leary DD. Regulation of area identity in the mammalian neocortex by Emx2 and Pax6. Science. 2000; 288:344–349. [PubMed: 10764649]

Brazel CY, Romanko MJ, Rothstein RP, Levison SW. Roles of the mammalian subventricular zone in brain development. Prog Neurobiol. 2003; 69:49–69. [PubMed: 12637172]

Bryja V, Cajanek L, Pachernik J, Hall AC, Horvath V, Dvorak P, Hampl A. Abnormal development of mouse embryoid bodies lacking p27Kip1 cell cycle regulator. Stem Cells. 2005; 23:965–974. [PubMed: 15941856]

Carey RG, Li B, DiCicco-Bloom E. Pituitary adenylate cyclase activating polypeptide anti-mitogenic signaling in cerebral cortical progenitors is regulated by p57Kip2-dependent CDK2 activity. J Neurosci. 2002; 22:1583–1591. [PubMed: 11880488]

Casaccia-Bonnefil P, Tikoo R, Kiyokawa H, Friedrich V Jr, Chao MV, Koff A. Oligodendrocyte precursor differentiation is perturbed in the absence of the cyclin-dependent kinase inhibitor p27Kip1. Genes Dev. 1997; 11:2335–2346. [PubMed: 9308962]

Coqueret O. Linking cyclins to transcriptional control. Gene. 2002; 299:35–55. [PubMed: 12459251]

Coskun V, Luskin MB. The expression pattern of the cell cycle inhibitor p19(INK4d) by progenitor cells of the rat embryonic telencephalon and neonatal anterior subventricular zone. J Neurosci. 2001; 21:3092–3103. [PubMed: 11312294]

Crockett DP, Burshteyn M, Garcia C, Muggironi M, Casaccia-Bonnefil P. Number of oligodendrocyte progenitors recruited to the lesioned spinal cord is modulated by the levels of the cell cycle regulatory protein p27Kip-1. Glia. 2005; 49:301–308. [PubMed: 15472992]

DiCicco-Bloom, E.; Sondell, M. Neural development and neurogenesis. In: Sadock, BJ.; Sadock, VZ., editors. Kaplan & Sadock's Comprehensive Textbook of Psychiatry. Philadelphia: Lippincott-Williams & Wilkins; 2005. p. 33-49.

Doetsch F, Verdugo JM, Caille I, Alvarez-Buylla A, Chao MV, Casaccia-Bonnefil P. Lack of the cell-cycle inhibitor p27Kip1 results in selective increase of transit-amplifying cells for adult neurogenesis. J Neurosci. 2002; 22:2255–2264. [PubMed: 11896165]

Durand B, Gao FB, Raff M. Accumulation of the cyclin-dependent kinase inhibitor p27/Kip1 and the timing of oligodendrocyte differentiation. Embo J. 1997; 16:306–317. [PubMed: 9029151]

Dyer MA, Cepko CL. p57(Kip2) regulates progenitor cell proliferation and amacrine interneuron development in the mouse retina. Development. 2000; 127:3593–3605. [PubMed: 10903183]

Dyer MA, Cepko CL. p27Kip1 and p57Kip2 regulate proliferation in distinct retinal progenitor cell populations. J Neurosci. 2001; 21:4259–4271. [PubMed: 11404411]

Elledge SJ, Harper JW. Cdk inhibitors: on the threshold of checkpoints and development. Curr Opin Cell Biol. 1994; 6:847–852. [PubMed: 7880532]

Franklin DS, Godfrey VL, Lee H, Kovalev GI, Schoonhoven R, Chen-Kiang S, Su L, Xiong Y. CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. Genes Dev. 1998; 12:2899–2911. [PubMed: 9744866]

Fujita N, Sato S, Tsuruo T. Phosphorylation of p27Kip1 at threonine 198 by p90 ribosomal protein S6 kinases promotes its binding to 14-3-3 and cytoplasmic localization. J Biol Chem. 2003; 278:49254–49260. [PubMed: 14504289]

Gotz M, Huttner WB. The cell biology of neurogenesis. Nat Rev Mol Cell Biol. 2005; 6:777–788. [PubMed: 16314867]

Gui H, Li S, Matise MP. A cell-autonomous requirement for Cip/Kip cyclin-kinase inhibitors in regulating neuronal cell cycle exit but not differentiation in the developing spinal cord. Dev Biol. 2007; 301:14–26. [PubMed: 17123502]

Ishizaki Y. Control of proliferation and differentiation of neural precursor cells: focusing on the developing cerebellum. J Pharmacol Sci. 2006; 101:183–188. [PubMed: 16823258]

Itoh Y, Masuyama N, Nakayama K, Nakayama KI, Gotoh Y. The cyclin-dependent kinase inhibitors p57 and p27 regulate neuronal migration in the developing mouse neocortex. J Biol Chem. 2007; 282:390–396. [PubMed: 17092932]

Joseph B, Wallen-Mackenzie A, Benoit G, Murata T, Joodmardi E, Okret S, Perlmann T. p57(Kip2) cooperates with Nurr1 in developing dopamine cells. Proc Natl Acad Sci U S A. 2003; 100:15619–15624. [PubMed: 14671317]

Kawaguchi A, Ogawa M, Saito K, Matsuzaki F, Okano H, Miyata T. Differential expression of Pax6 and Ngn2 between pair-generated cortical neurons. J Neurosci Res. 2004; 78:784–795. [PubMed: 15523634]

Kee N, Sivalingam S, Boonstra R, Wojtowicz JM. The utility of Ki-67 and BrdU as proliferative markers of adult neurogenesis. J Neurosci Methods. 2002; 115:97–105. [PubMed: 11897369]

Koguchi K, Nakatsuji Y, Nakayama K, Sakoda S. Modulation of astrocyte proliferation by cyclin-dependent kinase inhibitor p27(Kip1). Glia. 2002; 37:93–104. [PubMed: 11754208]

Lee MH, Reynisdottir I, Massague J. Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev. 1995; 9:639–649. [PubMed: 7729683]

Legrier ME, Ducray A, Propper A, Chao M, Kastner A. Cell cycle regulation during mouse olfactory neurogenesis. Cell Growth Differ. 2001; 12:591–601. [PubMed: 11751454]

Leibovitch MP, Kannengiesser C, Leibovitch SA. Signal-induced ubiquitination of p57(Kip2) is independent of the C-terminal consensus Cdk phosphorylation site. FEBS Lett. 2003; 543:125–128. [PubMed: 12753918]

Luskin MB, Coskun V. The progenitor cells of the embryonic telencephalon and the neonatal anterior subventricular zone differentially regulate their cell cycle. Chem Senses. 2002; 27:577–580. [PubMed: 12142335]

Matsuoka S, Edwards MC, Bai C, Parker S, Zhang P, Baldini A, Harper JW, Elledge SJ. p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene. Genes Dev. 1995; 9:650–662. [PubMed: 7729684]

Miyata T, Kawaguchi A, Saito K, Kawano M, Muto T, Ogawa M. Asymmetric production of surface-dividing and non-surface-dividing cortical progenitor cells. Development. 2004; 131:3133–3145. [PubMed: 15175243]

Moreno S, Farioli-Vecchioli S, Cerù MP. Immunolocalization of peroxisome proliferator-activated receptors and retinoid X receptors in the adult rat CNS. Neuroscience. 2004; 123:131–145. [PubMed: 14667448]

Muskhelishvili L, Latendresse JR, Kodell RL, Henderson EB. Evaluation of cell proliferation in rat tissues with BrdU, PCNA, Ki-67(MIB-5) immunohistochemistry and in situ hybridization for histone mRNA. J Histochem Cytochem. 2003; 51:1681–1688. [PubMed: 14623936]

Nagahama H, Hatakeyama S, Nakayama K, Nagata M, Tomita K. Spatial and temporal expression patterns of the cyclin-dependent kinase (CDK) inhibitors p27Kip1 and p57Kip2 during mouse development. Anat Embryol (Berl). 2001; 203:77–87. [PubMed: 11218061]

Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, Guillemot F. p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. Genes Dev. 2006; 20:1511–1524. [PubMed: 16705040]

Nishimori S, Tanaka Y, Chiba T, Fujii M, Imamura T, Miyazono K, Ogasawara T, Kawaguchi H, Igarashi T, Fujita T, Tanaka K, Toyoshima H. Smad-mediated transcription is required for transforming growth factor-beta 1-induced p57(Kip2) proteolysis in osteoblastic cells. J Biol Chem. 2001; 276:10700–10705. [PubMed: 11152674]

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR. Neurons derived from radial glial cells establish radial units in neocortex. Nature. 2001; 409:714–720. [PubMed: 11217860]

Noctor SC, Martinez-Cerdeno V, Ivic L, Kriegstein AR. Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. Nat Neurosci. 2004; 7:136–144. [PubMed: 14703572]

Ohnuma S, Philpott A, Wang K, Holt CE, Harris WA. p27Xic1, a Cdk inhibitor, promotes the determination of glial cells in Xenopus retina. Cell. 1999; 99:499–510. [PubMed: 10589678]

Paris M, Wang WH, Shin MH, Franklin DS, Andrisani OM. Homeodomain transcription factor Phox2a, via cyclic AMP-mediated activation, induces p27Kip1 transcription, coordinating neural progenitor cell cycle exit and differentiation. Mol Cell Biol. 2006; 26:8826–8839. [PubMed: 16982676]

Quintanilla-Martinez L, Davies-Hill T, Fend F, Calzada-Wack J, Sorbara L, Campo E, Jaffe ES, Raffeld M. Sequestration of p27Kip1 protein by cyclin D1 in typical and blastic variants of mantle cell lymphoma (MCL): implications for pathogenesis. Blood. 2003; 101:3181–3187. [PubMed: 12515730]

Ratineau C, Petry MW, Mutoh H, Leiter AB. Cyclin D1 represses the basic helix-loop-helix transcription factor, BETA2/NeuroD. J Biol Chem. 2002; 277:8847–8853. [PubMed: 11788592]

Reynaud EG, Leibovitch MP, Tintignac LA, Pelpel K, Guillier M, Leibovitch SA. Stabilization of MyoD by direct binding to p57(Kip2). J Biol Chem. 2000; 275:18767–18776. [PubMed: 10764802]

Reynaud EG, Pelpel K, Guillier M, Leibovitch MP, Leibovitch SA. p57(Kip2) stabilizes the MyoD protein by inhibiting cyclin E-Cdk2 kinase activity in growing myoblasts. Mol Cell Biol. 1999; 19:7621–7629. [PubMed: 10523650]

Rothschild G, Zhao X, Iavarone A, Lasorella A. E Proteins and Id2 converge on p57Kip2 to regulate cell cycle in neural cells. Mol Cell Biol. 2006; 26:4351–4361. [PubMed: 16705184]

Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol. 2000; 182:311–322. [PubMed: 10653597]

Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. 1995; 9:1149–1163. [PubMed: 7758941]

Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev. 1999; 13:1501–1512. [PubMed: 10385618]

Suh J, Lu N, Nicot A, Tatsuno I, DiCicco-Bloom E. PACAP is an anti-mitogenic signal in developing cerebral cortex. Nat Neurosci. 2001; 4:123–124. [PubMed: 11175869]

Sumrejkanchanakij P, Tamamori-Adachi M, Matsunaga Y, Eto K, Ikeda MA. Role of cyclin D1 cytoplasmic sequestration in the survival of postmitotic neurons. Oncogene. 2003; 22:8723–8730. [PubMed: 14647467]

Surjit M, Lal SK. Glycogen synthase kinase-3 phosphorylates and regulates the stability of p27kip1 protein. Cell Cycle. 2007; 6:580–588. [PubMed: 17351340]

Takahashi K, Nakayama K. Mice lacking a CDK inhibitor, p57Kip2, exhibit skeletal abnormalities and growth retardation. J Biochem (Tokyo). 2000; 127:73–83. [PubMed: 10731669]

Tanaka H, Yamashita T, Asada M, Mizutani S, Yoshikawa H, Tohyama M. Cytoplasmic p21(Cip1/WAF1) regulates neurite remodeling by inhibiting Rho-kinase activity. J Cell Biol. 2002; 158:321–329. [PubMed: 12119358]

Tsugu A, Sakai K, Dirks PB, Jung S, Weksberg R, Fei YL, Mondal S, Ivanchuk S, Ackerley C, Hamel PA, Rutka JT. Expression of p57(KIP2) potently blocks the growth of human astrocytomas and induces cell senescence. Am J Pathol. 2000; 157:919–932. [PubMed: 10980131]

Tury, A.; Sennvik, K.; DiCicco-Bloom, E. Society for Neuroscience. Atlanta: 2006 Oct. Cyclin-dependent kinase inhibitor overexpression promotes cell cycle exit and cell fate determination in embryonic rat cortical precursor cells.

Tuttle R, Nakagawa Y, Johnson JE, O'Leary DD. Defects in thalamocortical axon pathfinding correlate with altered cell domains in Mash-1-deficient mice. Development. 1999; 126:1903–1916. [PubMed: 10101124]

Urano T, Yashiroda H, Muraoka M, Tanaka K, Hosoi T, Inoue S, Ouchi Y, Toyoshima H. p57(Kip2) is degraded through the proteasome in osteoblasts stimulated to proliferation by transforming growth factor beta1. J Biol Chem. 1999; 274:12197–12200. [PubMed: 10212182]

van Lookeren Campagne M, Gill R. Tumor-suppressor p53 is expressed in proliferating and newly formed neurons of the embryonic and postnatal rat brain: comparison with expression of the cell cycle regulators p21Waf1/Cip1, p27Kip1, p57Kip2, p16Ink4a, cyclin G1, and the proto-oncogene Bax. J Comp Neurol. 1998; 397:181–198. [PubMed: 9658283]

Vercelli AF, Nucera C, Muzzi P, Tiveron C, Farsetti A, Moretti F, Della Pietra L, Mancini F, Sacchi A, Tatangelo L, Giaccari A, Trimarchi F, Vermiglio F, Pontecorvi A. Maternal thyroid hormones are transcriptionally active during embryonic development: results from a transgenic mouse model. Society for Neuroscience Abstract 697.7. 2007

Watanabe G, Pena P, Shambaugh GE 3rd, Haines GK 3rd, Pestell RG. Regulation of cyclin dependent kinase inhibitor proteins during neonatal cerebella development. Brain Res Dev Brain Res. 1998; 108:77–87.

Ye, W.; DiCicco-Bloom, E. Localization of cdk inhibitor p57kip2 between zones of proliferation and differentiation in the embryonic brain suggests roles in cell-cycle exit and differentiation In: Society for Neuroscience. Washington DC: Society for Neuroscience; 2005. (http://sfn.scholarone.com/itin2005/index.html)

Ye W, Mairet-Coello G, DiCicco-Bloom E. DNAse I pre-treatment markedly enhances detection of nuclear cyclin-dependent kinase inhibitor p57Kip2 and BrdU double immunostaining in embryonic rat brain. Histochem Cell Biol. 2007; 127:195–203. [PubMed: 17024454]

Zhang P, Liegeois NJ, Wong C, Finegold M, Hou H, Thompson JC, Silverman A, Harper JW, DePinho RA, Elledge SJ. Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature. 1997; 387:151–158. [PubMed: 9144284]

Zhang P, Wong C, DePinho RA, Harper JW, Elledge SJ. Cooperation between the Cdk inhibitors p27(KIP1) and p57(KIP2) in the control of tissue growth and development. Genes Dev. 1998; 12:3162–3167. [PubMed: 9784491]

Zhang P, Wong C, Liu D, Finegold M, Harper JW, Elledge SJ. p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. Genes Dev. 1999; 13:213–224. [PubMed: 9925645]

Zindy F, Cunningham JJ, Sherr CJ, Jogal S, Smeyne RJ, Roussel MF. Postnatal neuronal proliferation in mice lacking Ink4d and Kip1 inhibitors of cyclin-dependent kinases. Proc Natl Acad Sci U S A. 1999; 96:13462–13467. [PubMed: 10557343]

Zindy F, Quelle DE, Roussel MF, Sherr CJ. Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene. 1997a; 15:203–211. [PubMed: 9244355]

Zindy F, Soares H, Herzog KH, Morgan J, Sherr CJ, Roussel MF. Expression of INK4 inhibitors of cyclin D-dependent kinases during mouse brain development. Cell Growth Differ. 1997b; 8:1139–1150. [PubMed: 9372237]

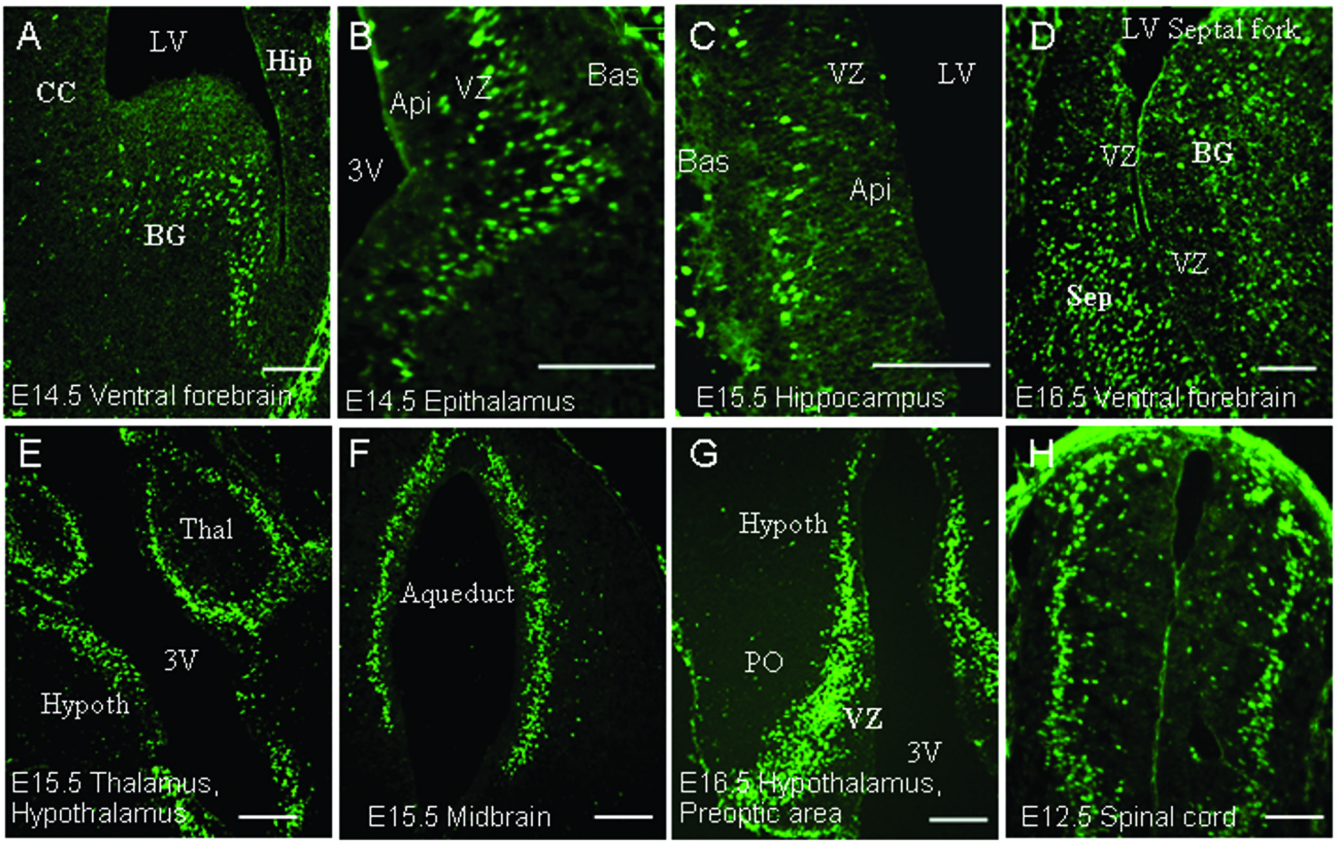

Figure 1.

Patterns of p57Kip2 immunoreactivity in the embryonic rat CNS. Coronal cryosections were obtained from a range of embryos (E12.5–16.5) and following DNase I pretreatment, were processed for p57Kip2 immunoreactivity with TSA enhancement (see methods; Ye et al, 2007). p57Kip2 expression in specific brain regions formed two distinct patterns consisting of an intermediate cell density with wide dispersion (A–D), or a more dense, laminar cell aggregate (E–H). A) Ventral forebrain, E14.5; B) Epithalamus, E14.5; C) Hippocampus, E15.5; D) Septum, basal ganglia, E16.5; E) Thalamus, hypothalamus, E15.5; F) Midbrain, E15.5; G) Hypothalamus, preoptic area, E16.5; H) Spinal cord, E12.5. Bar=100μm. 3V: third ventricle; Api: apical layer of ventricular zone; Bas: basal layer of ventricular zone; BG: basal ganglia; CC: cerebral cortex; Hip: hippocampus; Hypoth: hypothalamus; LV: lateral ventricle; PO: preoptic area; Sep: septum; Thal: thalamus; VZ: ventricular zone.

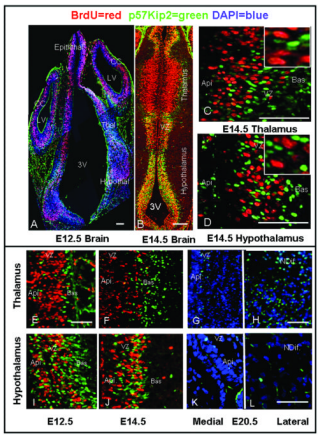

Figure 2.

Relationships of p57Kip2-expressing cells to BrdU labeled progenitors in embryonic brains during development. Embryos of various ages (E12.5 to E20.5) were processed for p57Kip2 and BrdU double immunolabeling 1–2 hr after maternal tracer injection. A) Coronal section of E12.5 forebrain indicates that p57Kip2 immunoreactivity is present throughout the dorsoventral and mediolateral extents of the CNS. B) The E14.4 diencephalon exhibits a striking variety of p57Kip2 and BrdU labeling patterns, particularly evident when comparing the thalamus to hypothalamus. C, D) Confocal images of E14.5 thalamus (C) and hypothalamus (D) are shown. Insets indicate no co-localization of BrdU and p57Kip2 in cell nuclei. p57Kip2 positive nuclei appear smaller than those exhibiting BrdU. E–H) in the thalamus, the zone of p57Kip2 immunoreactive cells lies primarily outside the region of BrdU labeled cells at E12.5 (E) and E14.5 (F). At E20.5, few positive cells lie adjacent to the ventricle (G) whereas there is abundant signal in the laterally placed thalamic differentiation region (H). I–L) In the hypothalamus, p57Kip2 positive cells are intermixed among the BrdU labeled cells forming a mosaic pattern in the proliferative layer at 12.5 (I) and E14.5 (J). At E20.5, p57Kip2 expression was minimal in the medial apical region (K) and was rarely observed laterally (L). Immunofluorescence was detected with anti-p57Kip2 (green), anti-BrdU (red) and DAPI staining (blue). 3V: third ventricle; Api: apical layer; Bas: basal layer; Epithal: epithalamus; NDif: neuron differentiation zone: VZ: ventricular zone. Bars=50μm.

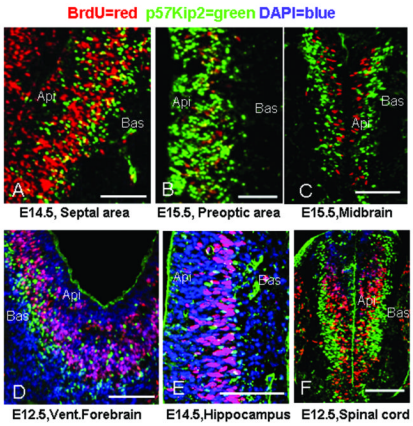

Figure 3.

Distinct patterns of p57Kip2 expression are observed during embryonic development. Embryos of various ages (E12.5 to E15.5) were processed for p57Kip2 and BrdU double immunolabeling 1–2 hr after maternal tracer injection. The wide dispersion, mosaic pattern of p57Kip2 and BrdU expression was observed in septum (A), ventral forebrain (D) and hippocampus (E). The dense laminar aggregate pattern was observed in the preoptic area (B), midbrain (C) and spinal cord (F). Immunofluorescence was detected with anti-p57Kip2 (green), anti-BrdU (red) and DAPI staining (blue). Api: apical layer; Bas: basal layer. Bars=100μm.

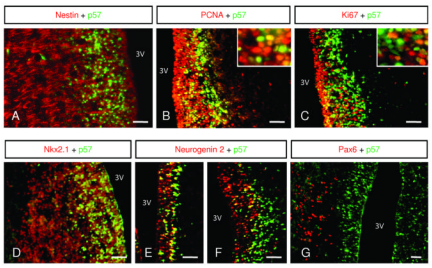

Figure 4.

Relationships of p57Kip2 expression to markers of developmental stage and regional identity in the E14.5 diencephalon. A–D) Basal hypothalamus. In the basal hypothalamus, p57Kip2 expressing cells lie medially within the broad nestin positive area (A). The majority of these cells co-localize p57Kip2 immunoreactivity with the proliferative marker, PCNA (B). In contrast, only 50% of them co-express the proliferative marker, Ki67 (C). p57Kip2 positive cells co-express the transcription factor, Nkx2.1. E, F) The proneuronal gene neurogenin 2 exhibits minor co-localization with p57Kip2 in the upper hypothalamus (E) and in the thalamus (F). G) In the thalamus, there is no co-localization of p57Kip2 with Pax6. Bars=50μm.

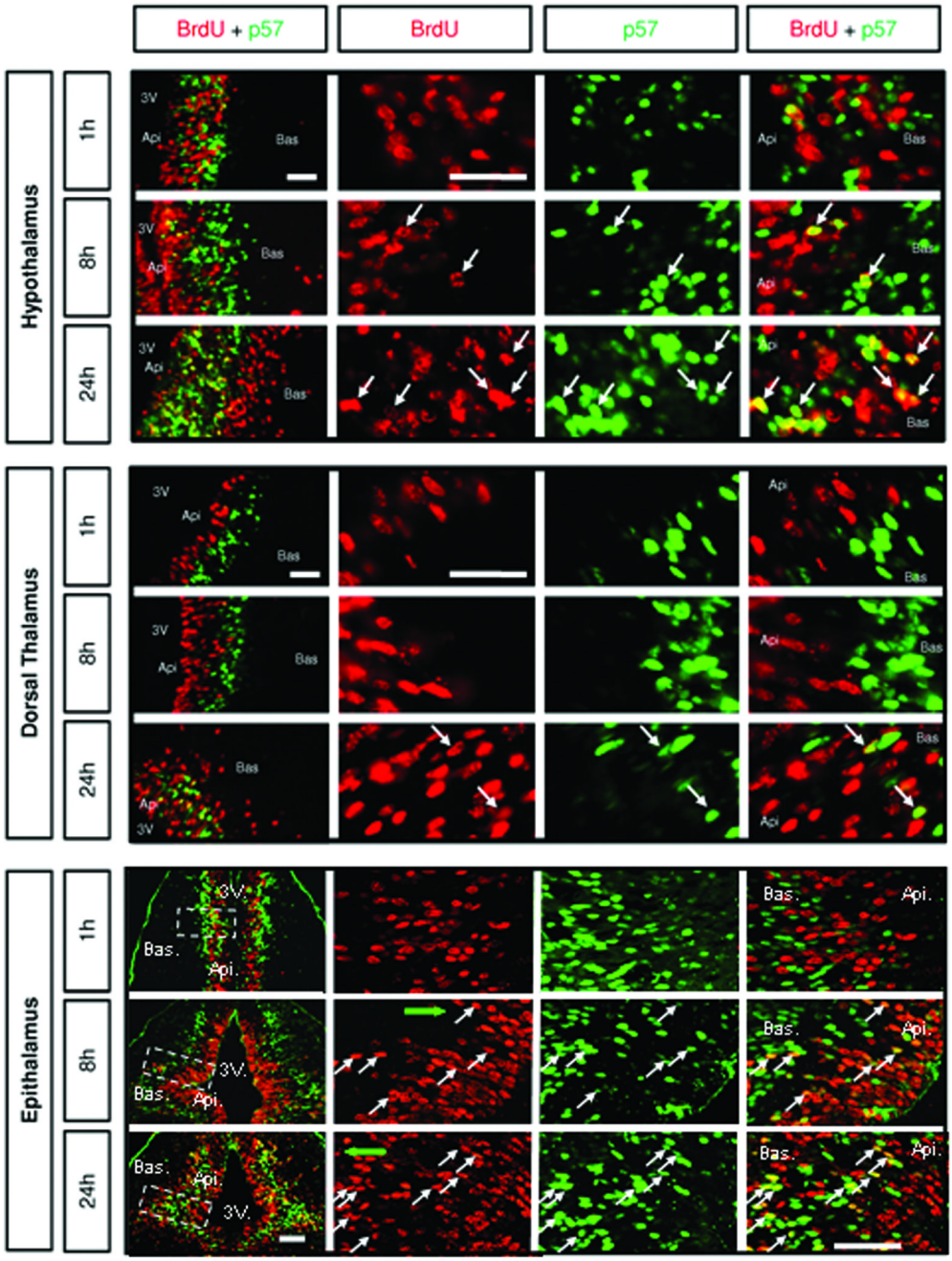

Figure 5.

Temporal relationships of p57Kip2 expression to S phase entry in the E14.5 diencephalon. Embryos were fixed at 1, 8 and 24 hr after BrdU injection into dams at E14.5 and processed for double immunolabeling of p57Kip2 expression and BrdU incorporation in the hypothalamus (top), dorsal thalamus (middle) and epithalamus (bottom). In the left BrdU + p57 column, low magnification images show the relationship of S phase cells to p57Kip2 expression in each region, and demonstrate migration of BrdU labeled cells from the initial basal location at 1 hr to a more apical position by 8 hr. High magnification images show individual labeled nuclei, and white arrows identify doubled immunolabeled cells. (Top) In the hypothalamus, p57Kip2-expressing cells exhibit BrdU co-labeling at 8 hr, with a further

increase at 24 hr. (Middle) In the dorsal thalamus, double labeling is absent or very rare at 8 hr, and present by 24 hr. (Bottom) In the epithalamus, double labeled cells are common at 8 hr, at a time when the BrdU labeled precursors have migrated to the apical region adjacent to the ventricle (green arrow) from the initial basal location at 1 hr. At 24 hr, many BrdU labeled cell have migrated toward the basal margin (green arrow) and p57Kip2/BrdU double labeling further increases. Immunofluorescent signals include p57Kip2 (green), BrdU (red) and DAPI (blue). White arrows indicate co-localization of BrdU and p57Kip2 signals. Green arrows indicate direction of BrdU labeled cell migration. Api: apical layer; Bas: basal layer. Bar: 50μm in (A), 100μm in (B).

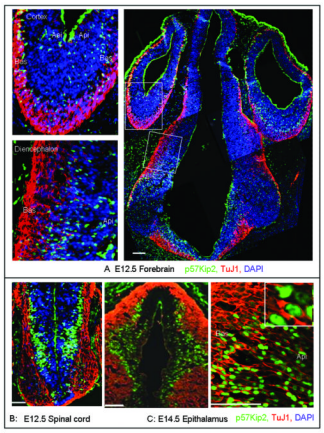

Figure 6.

Relationship of p57Kip2 expression to neuronal differentiation marker, TuJ1, in the developing CNS. Embryos were fixed at 1h after BrdU injection into dams at E12.5 (A, B) and E14.5 (C) and processed for double immunolabeling of p57Kip2 and TuJ1 expression in the cortex and diencephalon (A), spinal cord (B) and epithalamus (C). p57Kip2 signal is detected at the transitional region between the proliferation zone and cells expressing TuJ1. Immunofluorescent signals include p57Kip2 (green), BrdU (red) and DAPI (purple). Images in (C) were obtained using confocal microscopy. Bars=50μm.

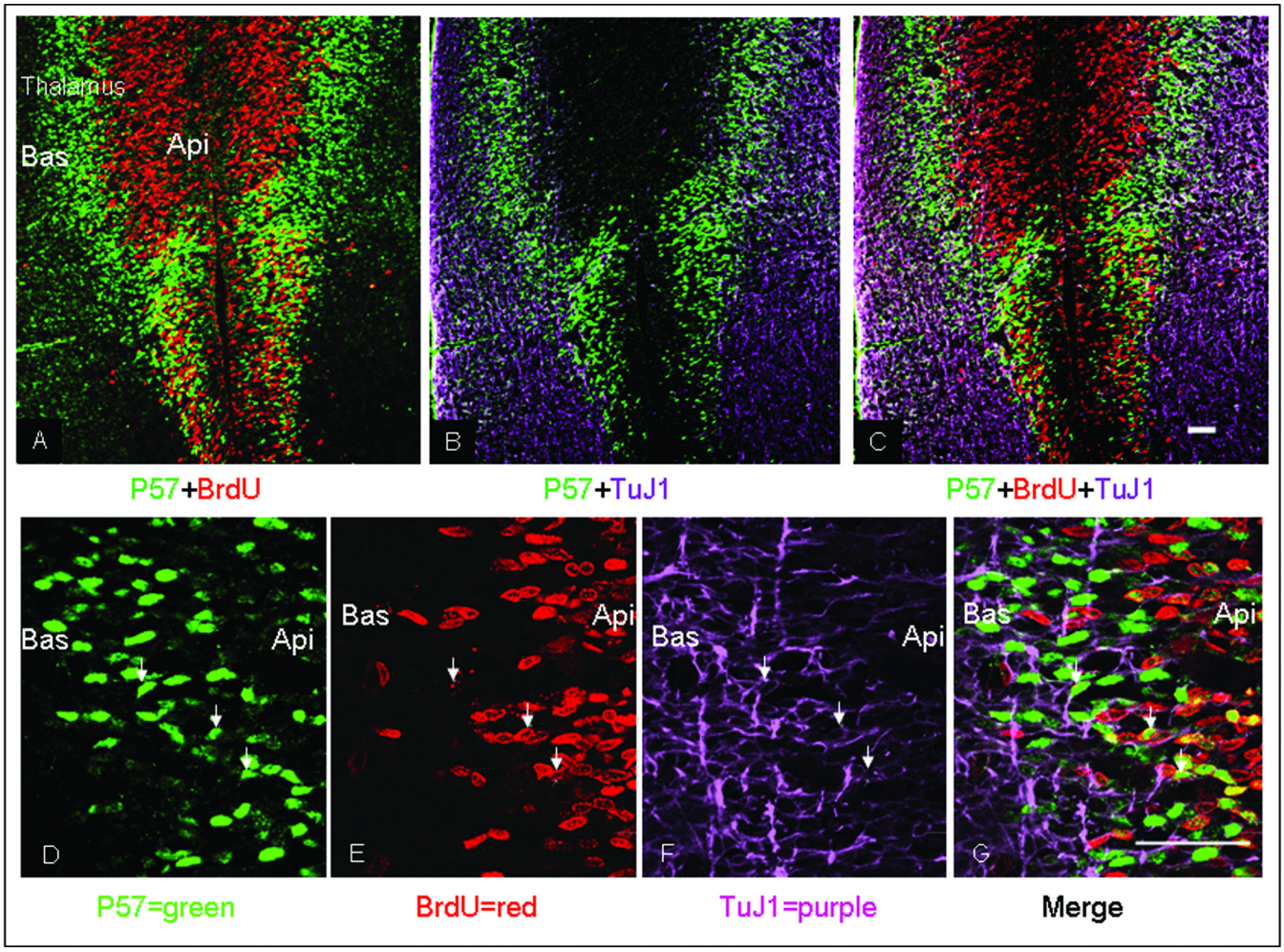

Figure 7.

Temporal relationships of p57Kip2 expression to S phase and neuronal differentiation in E14.5 diencephalon. Embryos were fixed at 2 hr (A–C) and 8 hr (D–G) after BrdU injection into dams at E14.5 and processed for triple immunolabeling of p57Kip2, TuJ1 and BrdU. The double (A, B) and triple (C) labeling indicate the spatial distribution of p57Kip2, BrdU and TuJ1 positive cells. Confocal image (D–G) analysis indicates the co-localization of p57Kip2, TuJ1 and BrdU in new neurons at 8h after BrdU injection (arrows). Immunofluorescent signals include p57Kip2 (green), BrdU (red) and DAPI (purple). Api: apical layer; Bas: basal layer. Bars=50 μm.

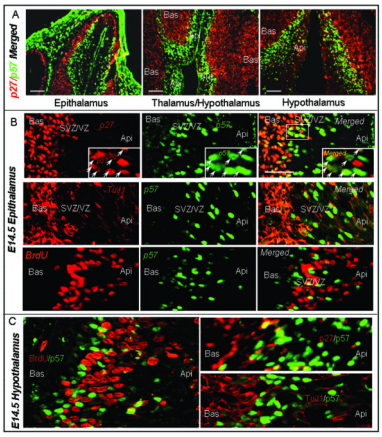

Figure 8.

Spatial relationship of p27Kip1 and p57Kip2 expression in the E14.5 diencephalon. Double immunolabeling of p57Kip2 (green) and p27Kip1 (red) or TuJ1 (red) was performed on adjacent sections. (A) p57Kip2 was expressed primarily in the proliferative zone whereas p27Kip1 signal was localized more laterally. (B) Confocal analysis of the epithalamus indicates that p27Kip1 is expressed in the neuronal differentiation zone (NDf) adjacent to the VZ/SVZ zone exhibiting p57Kip2 signal (upper panels). A subset of cells co-localize p27Kip1 and p57Kip2 (inset, arrows). TuJ1 appears to be localized to the same region as p27Kip1 (middle panels). Many p57Kip2-expressing cells co-localize TuJ1 in the boundary region. The epithalamus exhibits dispersed p57Kip2 expressing cells in a mosaic pattern with BrdU labeled precursors (lower panels). (C) The hypothalamus also exhibits a mosaic pattern of p57Kip2 and BrdU expressing cells (left) and p57Kip2-expressing cells co-label with both p27Kip1 and TuJ1 (right). Api: apical region; Bas: basal region. Bars=50μm.

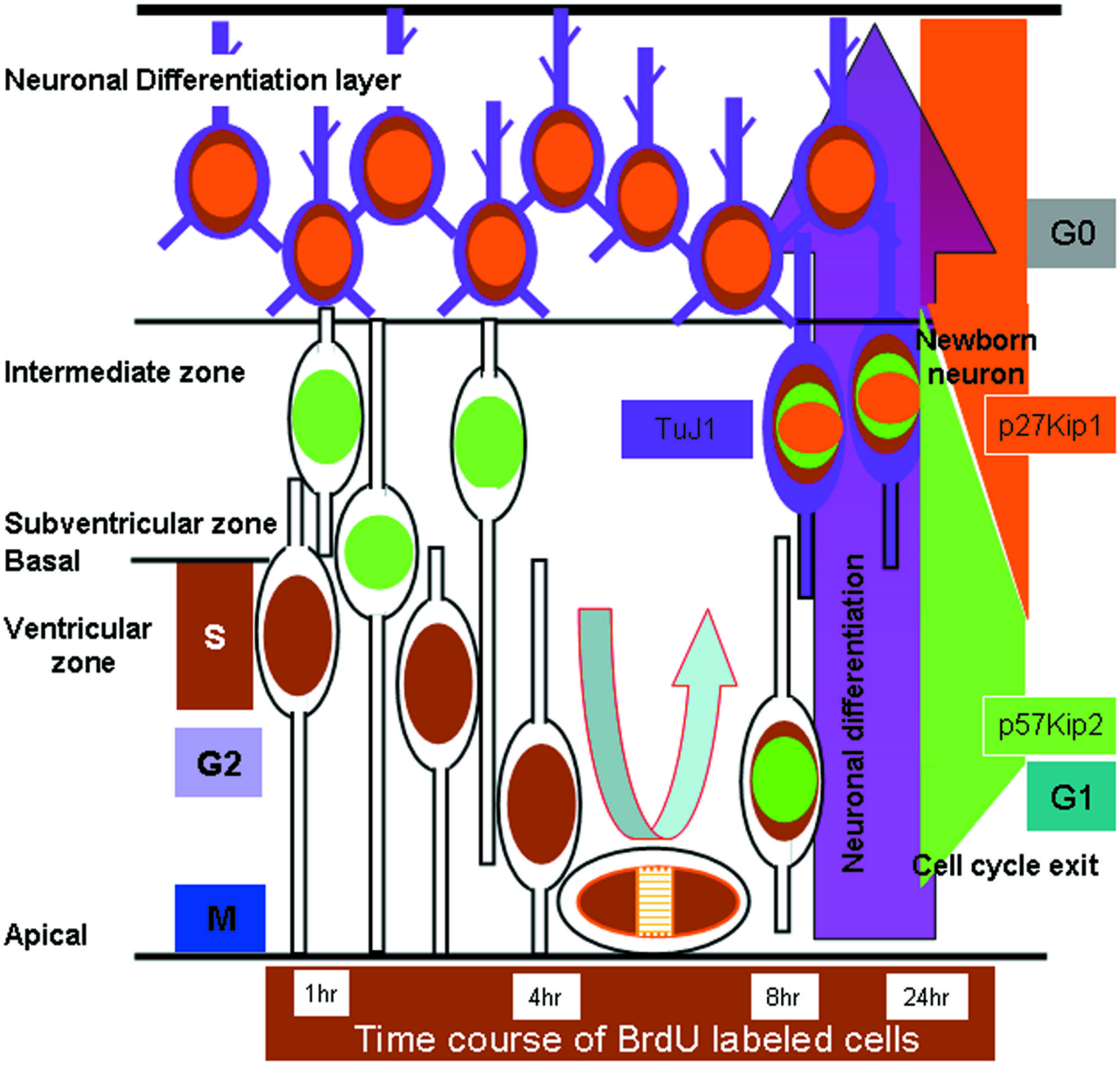

Figure 9.

Model of p57Kip2 protein expression during embryonic development. As cells complete mitosis on the apical border of the VZ, a subset will begin to express p57Kip2 approximately 8 hours after S phase, when cells are making the decision to exit the cycle during G1 phase. The expression of p57Kip2 will be co-incident with the onset of differentiation, including expression of cytoskeletal protein, β-III tubulin (TuJ1). As postmitotic neurons migrate away from the VZ into the intermediate zone, p57Kip2 expression diminishes and is followed by p27Kip1, which may plays roles in maintaining the postmitotic state and/or in cell migration and differentiation.

Table 1

Patterns of p57Kip2 immunoreactivity in developing embryonic rat forebrain

Forebrain	E12.5	E14.5	E15.5	E16.5	E18.5	E20.5
Olfactory bulbs		X	X	X	X	
Olfactory bulb **dif. fld**.			XX	X		
Cerebral Cortex						
Neocortical **neuroepithe**	X	X	X	X	X	
Cingulate cortex **neuroepithe**	X	X	X	X	X	
Neocortex	X	X	X	X	X	X
Hippocampus						
Neuroepithelium (neuroepithe.)						
Hippocampal **neuroepithe**	X	XX	XX	X	X	
Subicular **neuroepithe**	X	XX	XX	X	X	
Dentate gyrus **neuroepithe**	X	XX	XX	X	X	
Differentiation field (dif. fld.)						
Hippocampus **dif. fld**.		X	X	X	XX	X
Dentate gyrus **dif. fld**.		X	X	XX	X	X
Amygdala						
Amygdaloid **neuroepithe**	XX	XX	XX	XX	X	
Amygdaloid **SVZ**	X	X	X	X	X	X
Amygdala **dif. fld**.		X	X	X	XX	XX
Preoptic area & optic epithe.						
Preoptic **neuroepithe**.	X	XX	XXX	XX		
Eye stalk epithe		XXX	XX	XX		
Preoptic area **dif. fld**.		XX	XX	XXX	X	
Medial preoptic nuclear		XX	XX	XX	X	
Basal Ganglia						
Striatal & Pallidal **neuroepithe**	X	X	X	XX		
Strionuclear **neuroepithe**	XX	XX	XX	X	X	X
Striatal & Pallidal **SVZ**		XX	XX	XX	X	X
Pallidium **dif. fld**.			XX	XX		
Striatum **dif. fld**.			X	XX	X	

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Forebrain	E12.5	E14.5	E15.5	E16.5	E18.5	E20.5
Bed nucleus of stria terminalis		X	X	XX	X	X
Nucleus accumbens **dif. fld**.					X	X
Septal Area						
Septal **neuroepithe**	X	XX	XXX	XX	XX	X
Septal **SVZ**					XX	X
Septum **dif. fld**.		XX	XX	XX	XX	
Medial septum		X	X	XXX	X	
Rhinencephalon						
Rhinencephalic **neuroepith**.	XX	X				
Rhinencephalon **dif. fld**.		X	X	X	X	X
Piriform cortex		X	X	X	X	X
Thalamus						
Neuroepithelium						
Epithalamic **neuroepithe**.	X	XX	XX	XX	X	X
Anterior thalamic **neuroepithe**	X	XX	XX	XX	X	X
Intermediate thalamic **neuroepithe**	X	X	X			
Posterior thalamic **neuroepithe**.		X	XX	XX		
Differentiation field						
Epithalamus **dif. fld**.		X	X	XX	X	
Intermediate thalamic **dif. fld**.		X	XX	XX	XXX	XXX
Posterior thalamus **dif. fld**.				X	XXX	XXX
Lateral habenular				XX	XX	
Ventral medial nucle.				XXX	XXX	XX
Dorsal & ventral lateral genciulate nucle.					XX	XX
Hypothalamus						
Hypothalamic **neuroepithe**.	XX	XX	XX	XX	X	X
Fields of Forel **dif. fld**.	X	X	X	X		
hypothalamus **dif. fld**.		0	X	XX	X	X
Anterior nucleus				XX	XXX	

Note: Coronal cryosections obtained from a range of embryos (E12.5–20.5) were processed for p57Kip2 immunoreactivity with TSA enhancement (see methods). p57Kip2 immunoreactivity in specific brain regions demonstrated no detectable signal (blank), or exhibited a pattern consisting of low cell density (x), intermediate cell density (xx) that was widely dispersed, or a more dense laminar aggregation (xxx). SVZ: Subventricular Zone; dif.fld: Differentiation Field; neuroepithe: Neuroepithelium.

Table 2

Percentage of cells in the proliferative (Prolif) and non-proliferative (Non-P) regions of the E14.5 diencephalon that express p57Kip2 and BrdU.

Structure	Region	Percent (%) BrdU+ cells	Percent (%) p57+ cells
Dorsal Thalamus (3874*)	Prolif (P)	49.7 ± 15.3	3.5 ± 1.9
	Non-P	2.4 ± 1.2	63.2 ± 4.2
Hypothalamus (3330)	Prolif	15.4 ± 3.6	52.2 ± 3.1
	Non-P	0.9 ± 0.05	2.4 ± 0.5

Note: Two hours after maternal BrdU injection, E14.5 embryos were processed for combined p57Kip2 and BrdU immunolabeling and DAPI nuclear staining. At least 2 sections from each of 2–3 embryos per region were assessed using confocal imaging at 400X magnification for the percent of cells exhibiting each signal, expressed as a ratio to total cells visualized by DAPI staining. Data are expressed as mean ± SD.

* indicates the total number of cells counted for each structure. The proliferative region was identified by BrdU labeling and DAPI morphological features, while the non-proliferative region was lateral to this.

Table 3

Distribution of p57Kip2 and p27Kip1 positive cells in the proliferative (Prolif) and non-proliferative (Non-P) regions of the E14.5 diencephalon.

Structure	Region	p57+ Cell Distribution (%)	p27+ Cell Distribution (%)	p27+ p57+ per total p57+ Cell
Epithalamus (2482*)	Prolif (P)	73.8 ± 9.5	20.7 ± 3.2	34.3 ± 2.1
	Non-P	26.2 ± 4.7	79.3 ± 6.7	48.5 ± 4.6
Dorsal Thalamus (3874)	Prolif	4.4 ± 2.0	32.3 ± 2.5	12.2 ± 1.8
	Non-P	95.6 ± 2.0	67.7 ± 2.5	73.0 ± 2.6
Hypothalamus (3330)	Prolif	92.2 ± 1.1	21.0 ± 1.8	68.1 ± 4.6
	Non-P	7.8 ± 1.1	79.1 ± 4.6	7.6 ± 1.0

E14.5 embryos were processed for combined p57Kip2 and p27Kip1 immunolabeling and DAPI nuclear staining. At least 2 sections from each of 2–3 embryos per region were assessed using confocal imaging at 400X magnification for the distribution of positive cells between the proliferative and non-proliferative regions in each structure. The percent of p57Kip2-expressing cells that also co-labeled for p27Kip1 was calculated as a ratio to total p57Kip2 cells. Data are expressed as mean ± SD.

* indicates the total number of cells counted for each structure.