

# FGF2 Maintains the Proliferation of Neural Progenitors by Actively Suppressing the CKI p27Kip1 through Regulation of Cks1b Transcription

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# FGF2 MAINTAINS THE PROLIFERATION OF NEURAL PROGENITORS BY ACTIVELY SUPPRESSING THE CKI P27<sup>KIP1</sup> THROUGH REGULATION OF CKS1B TRANSCRIPTION

By

Andrew J. Darr

A DISSERTATION

Submitted to the Faculty of the University of Miami in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Coral Gables, Florida

December 2009

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## UNIVERSITY OF MIAMI

## A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Identifying the mechanisms that regulate neural precursor cell (NPC) proliferation

and differentiation is important for understanding CNS development among different vertebrates. My work has focused specifically on understanding how mitogenic factors, like basic fibroblast growth factor (FGF2), regulate the NPC cell cycle. Mitogenic factors and serum are thought to drive cell cycle and therefore proliferation mainly by activating G1-type cyclin-dependent kinases (CDKs). The general hypothesis being addressed here is that FGF2 also promotes cell cycle progression of NPCs through the degradation of the cell cycle inhibitor p27<sup>Kip1</sup>. I show that, in the presence of FGF2 in vitro, embryonic rat cortical NPCs express high protein levels of the CDC28 protein kinase regulatory subunit 1b (Cks1b), a component of the SCF<sup>Skp2</sup> E3 ubiquitin ligase complex that targets p27<sup>Kip1</sup> for proteasomal degradation. I also show that NPCs maintained in FGF2 express undetectable levels of p27KIP1, while removal of FGF2 results in increased p27<sup>Kip1</sup> protein expression and decreased protein expression of Cks1b. RNA expression data shows that Cks1b mRNA is reduced in non-dividing NPCs but is present in dividing NPCs, suggesting that Cks1b is being regulated at the transcriptional

level. Analysis of the putative promoter of Cks1b reveals numerous conserved transcription factor consensus sites that could potentially play a role in regulation of Cks1b transcription, including consensus sites for E2F and the cell cycle-dependent element (CDE) cell cycle genes homology region (CHR) tandem repressor element. I use chromatin immunoprecipitation and luciferase assays to identify which E2Fs occupy and regulate the transcription of Cks1b under different conditions of mitogen stimulation. The data show that E2F4 occupies the promoter of Cks1b in non-dividing NPCs while E2F1 binds exclusively in proliferating NPCs. Mutation of either the E2F or CDE/CHR consensus sites independently de-represses the activity of a Cks1b promoter reporter in NPCs in G0/G1, while mutation of both sites delays induction of promoter activity. Finally, I use in ovo electroporation to determine if p27Kip1 has an additional role in neuronal differentiation during early spinal cord development. I show that ectopic expression of p27Kip1 is insufficient to induce neuronal differentiation in spinal cord progenitors.

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#### Chapter I

#### **GENERAL INTRODUCTION**

#### I. METAZOAN DEVELOPMENT

The development of a multicellular organism from a single fertilized egg cell follows a specific design plan that incorporates cell proliferation, growth, cytodifferentiation, programmed cell death, and patterning. At the core of these fundamental developmental processes is the cell cycle, which is regulated by multiple developmental signaling pathways and can be modified to meet the demands of a cell at specific developmental stages. For example, during the early stages of embryogenesis, the cell cycle is abbreviated to encourage rapid proliferation and expansion of pools of undifferentiated precursor cells, but a progressively longer cell cycle is adopted at later stages of development as more cells become differentiated and are incorporated into tissues (Sidman et al. 1959; Fujita 1962; Kauffman, 1967; Wilson 1972; von Waechter and Janesch 1972; Schultze et al. 1974; Kornack and Rakic 1998). The difference in cell cycle length is primarily due to the inclusion and duration of the first gap phase, called G1 that integrates multiple growth or differentiation cues that facilitate either cell cycle reentry or withdrawal, respectively (von Waechter and Janesch 1972; Wilson 1972; Takahashi et al., 1995; Miyama et al. 1997). With respect to early CNS development, undifferentiated neural stem cells gradually slow their cell cycle division by increasing the time spent in G1 in order to accommodate a multitude of instructive cues that determine the timing of their final mitosis as well as their commitment to a particular lineage.

#### II. NEURAL STEM CELLS AND CNS DEVELOPMENT

Stem cells are defined by their ability to self-renew by giving rise to identical copies of themselves and the capacity to give rise to one or more differentiated cell types. Neural stem cells (NSC) are multipotent and are distributed throughout the entire rostrocaudal axis of the neural tube. Neural stem cells, as well as more restricted progenitors, were first identified in the developing cortex through in vivo lineage studies using retroviral vectors (Luskin et al., 1988; Price and Thurlow, 1988). In the CNS, as with other tissues, lineage-restricted progenitors (LRP) have been identified as bipotent precursors and can give rise to oligodendrocytes and astrocytes, or neurons and oligodendrocytes, or unipotent, generating a single neural cell type (He et al., 2001; Lee et al., 2000; Luskin et al., 1988, 1993; Price and Thurlow, 1988; Raff et al., 1983; Williams and Price, 1995). While multipotent neural stem cells have been characterized by numerous groups in vitro, the capacity for a single NSC to generate all three neural cell types in vivo has been controversial (Anderson, 2001).

During the early stages of embryogenesis, the mammalian CNS is composed almost exclusively of a single layer of pseudostratified neuroepithelium. Most of the neurons and neuroglia that will make up the CNS are ultimately derived from these neuroepithelial cells (either directly or indirectly through intermediate progenitors) and can therefore be referred to as neural stem cells (Merkle & Alvarez-buylla, 2006). These bipolar neuroepithelial cells undergo mitosis exclusively at the ventricular (apical) surface. Their nuclei undergo cell cycle-dependent apical-to-basal migration during G1 and basal-to-apical movement during G2, a process called interkinetic nuclear migration

(Sauer, 1935) (Figure 1 A). This back and forth movement of the nucleus results in the pseudostratification of the neuroepithelium, where various nuclei are positioned in several layers depending on the phase of the cycle they happen to be passing through, but all cells extend from the basal lamina to the ventricle (Sauer, 1935). Prior to the onset of neurogenesis, the entire neuroepithelium consists of a single germinal layer, the ventricular zone (VZ). Within the VZ, neuroepithelial precursors undergo either symmetric or asymmetric cell division (Huttner & Kosodo, 2005). Precursors that undergo symmetric cell division produce two progenitor cells, each capable of selfrenewal and contribute to the pool of undifferentiated cells (Potten & Loeffler, 1990). Coinciding with the onset of neurogenesis, many of the divisions become either asymmetric, producing one daughter cell that continues to divide and proliferate and one that is committed to become a neuron or neuroglial cell, or symmetric, producing two differentiated progeny (Kosodo et al., 2004; Chenn, 2005; Gotz & Huttner, 2005). In response to extracellular cues, neuroepithelial precursors eventually undergo a terminal mitosis and give rise to differentiated progeny that lose their attachment to the basal lamina and migrate out of the VZ to populate the cortex and spinal cord (Hatten, 1999) (Figure 1 B).



Schematic Representation of Germinal and Cellular zones in the Developing Mouse Spinal Cord

(B)



**Figure 1.** Neurogenesis in the developing neural tube. (A) During the cell cycle, the nuclei of proliferating neuroepithelial cells translocate from the apical/ventricular surface outward toward the basal/mantle surface during G1 and return to the apical surface during S and G2. In late S phase, the peripheral process is withdrawn and mitosis, or M phase of the cell cycle, occurs at the ventricular surface. Following cytokinesis, the distal process is again extended, and the cycle begins again. At some point, cells undergo terminal mitosis and cease further synthesis of DNA, becoming arrested in G1 phase of

the cell cycle. Post-mitotic cells then begin to migrate toward the basal lamina and past the nuclei of the neuroepithelial cells (Adapted from F. C. Sauer, J. Comp. Neurol 62:377-405 (1935)). (B) In the early neural tube, the region between the ependymal, or ventricular surface and the mantle zone consists primarily of neuroepithelial precursor cells at different phases of the cell cycle. Prior to the onset of neurogenesis, neural progenitors begin dividing asymmetrically to give rise to both a self-replicating progenitor and a differentiated neuron, the latter migrates away from the ventricular zone toward the basal lamina. Prior to the onset of neurogenesis, neural progenitors begin dividing asymmetrically to give rise to both a self-replicating progenitor and a differentiated neuron. As more progenitors undergo neurogenic divisions, post-mitotic cells begin to migrate radially outward from the ventricular zone (VZ) past the nuclei of neuroepithelial cells toward the basal lamina forming the marginal zone (MZ) that increases in thickness as the neural tube differentiates into the spinal cord. With the accumulation of post-mitotic cells in the MZ, an intermediate zone (IZ) is formed between the MZ and VZ that also increases in thickness as development proceeds. In the MZ, differentiated post-mitotic neurons that accumulate there integrate themselves into functional neuronal circuits. Later in development, the mantle zone accumulates postmitotic neurons that form functional neuronal circuits. Eventually, the ventricular zone is reduced to a thin layer of precursor cells surrounding the central canal. PVE =pseudostratified ventricular epithelium; V = ventricular zone; I = intermediate zone; M = mantle zone; E = embryonic day (Adapted from M. Jacobson, Developmental Neurobiology 1978 Plenum Presss, 2nd edition).

Neural stem/progenitor cells in the embryonic cerebral cortex can be categorized into two groups based on their location within the PVE at the time of cell division. The first group consists of neural stem/progenitor cells that undergo mitosis at or in close proximity to the apical/ventricular surface and that, during M-phase, are connected to each other by adherens junctions at the apical-most end of their lateral plasma membrane. These are the neuroepithelial cells, and radial glial cells in the cortex, which can also be called apical progenitors (APs). The second group consists of neural progenitors that undergo mitosis toward the basal aspect of the PVE, typically in the basal VZ and SVZ and that, during M-phase, are no longer part of the adherens junctionconnected AP assembly. These are called basal or intermediate progenitors. This latter group divides symmetrically to give rise to differentiated progeny almost exclusively and contributes significantly to patterning of CNS structures (Farkas and Huttner, 2008). The majority of intermediate/basal progenitors are found in the telencephalon, where they form the subventricular zone (SVZ), a mitotic layer that is basal to the ventricular zone (VZ) (Haubensak et al. 2004). Intermediate progenitors exist only in specific areas of the mammalian brain, and are only rarely observed in the hindbrain or spinal cord (Haubensak et al. 2004; Smart 1972, 1973). They are most abundant in the ventral telencephalon where at midneurogenesis they make up more than half of all actively dividing cells (Haubensak et al. 2004). By comparison, intermediate/basal progenitors never exceed more than 25% of all dividing cells in the dorsal telencephalon at any point during neurogenesis (Haubst et al. 2004; Miyata et al. 2004). At the peak of neurogenesis, radial glial cells constitute the majority of VZ progenitors in developing mouse brain, especially in areas where the number of basal progenitors is small (Hartfuss et al. 2001; Mori et al. 2005). In fact, the high concentration of intermediate progenitors and increase in SVZ is associated with enhanced neurogenesis and neocortical expansion in primate brain evolution (Kornack and Rakic, 1998; Smart et al. 2002). In the adult, neural stem cells have been identified in discrete regions of high-density mitotic activity, namely the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus within the hippocampal formation (Alvarez-buylla & Garcia-Verdugo, 2002).

#### III. NEUROGENESIS AND THE CONTROL OF THE CELL CYCLE

Precise regulation of the proliferation of undifferentiated precursors is necessary to ensure that the developing CNS is the appropriate overall size, and cell cycle withdrawal must be coordinated with cell fate specification to ensure that the appropriate number of each neural cell type is generated (Sommer & Rao, 2002). A premature increase in cells exiting the cell cycle during development would deplete the precursor pool before histogenesis is complete, and since NSCs generate neurons and neuroglia in an evolutionarily conserved sequence over the course development (Bayer & Altman, 1991; Qian et al., 1998), it would result in an increased proportion of early-born cell types at the expense of later-born cell types, disrupting subsequent processes like cell migration and patterning (Caviness et al., 1995). Thus, regulation of the cell cycle of neural precursors is a critical parameter in formation of the CNS. Over the course of CNS development, the overall duration of the cell cycle increases, which is attributable to a lengthening of the G1 phase of the cycle (Takahashi et al., 1995) (Figure 2 A). In mouse cortical progenitor cells, the M and G2 phases of the cell cycle barely change from embryonic days E10 to E19, while the G1 phase nearly triples in length (Takahashi et al., 1995). Mouse cortical precursor cells treated with differentiation factors show an increase in the duration of the G1 phase. Conversely, treatment with mitogenic factors decreases G1 length (Lukaszewicz et al., 2002). In the rat cortex, neurogenesis occurs between embryonic days E12 and E20 (Bayer & Altman, 1991), a time period when rat neural precursors increase their cell cycle time from 11 hours (E12) to 19 hours (E18) (Cavanagh et al., 1997). The gradual increase in the length of the cell cycle reflects the

transition from an early proliferative state to a more neurogenic state (Caviness et al., 1995; Calegari & Huttner, 2003) (Figure 2 B). The lengthening of the G1 phase corresponds to an increased receptivity to differentiation signals, and a number of multifunctional growth factors are known to affect G1 cell cycle components (Baek et al., 2003; Kioussi et al., 2002; Oliver et al., 2003). This has led to speculation that an abbreviated G1 phase might safeguard stem cells from differentiation factors thereby maintaining their undifferentiated, proliferative state (Mummery et al., 1987; Dehay & Kennedy, 2007).

(A)



**Figure 2.** The overall length of the cell cycle increases during embryogenesis. (A) The values for the cell cycle parameters for E11-E16 in mouse (Takahashi et al., 1995) and rat (von Waechter and Janesch, 1972), obtained experimentally by cumulative labeling with bromodeoxyuridine, represent average values taken stepwise from a limited time of sampling for each of the respective days. Tc - Ts, the experimental interval for each day, lengthens over the course of neurogenesis. The length of the overall cell cycle (Tc) is approximately doubled over the interval E11-E16 with this increase due to increase in the length of G1 phase. There is no systematic change in either the length of S phase or combined G2 and M phases (TG2+M) over the same interval (Adapted from T. Takahashi et al. J. Neurosci. 15:6046 (1995)). (B) The generation of neurons, astrocytes, and oligodendrocytes by neural progenitors follows an intrinsically programmed sequence consisting of temporally distinct yet overlapping periods of histogenesis. In rate, neurogenesis peaks at embryonic day 14 (E14), astrocytogenesis peaks at postnatal day 2 (P2), and oligodendrocytogenesis peaks at P14 (Adapted from C.M. Sauvageot et al. Curr. Opin. Neurobiol. 12(3):244 (2002)).

#### IV. EXTRINSIC SIGNALING MOLECULES

#### A. Multifunctional Growth Factors in CNS Development

The number and neuronal identity of progeny generated by a neural precursor cell in the ventricular zone depends on its regional distribution within the neural tube as well as its location within a particular developmental period (Tanabe and Jessell, 1995). Several families of multifunctional growth factors have been implicated in CNS patterning and growth including Wingless/nt-related (WNT), Bone morphogenetic protein (BMP), and Sonic hedgehog (SHH) signaling molecules. The formation of concentration gradients emanating from opposite poles of the neural tube, with WNTs and BMPs originating from the dorsal side and SHH from the ventral side, control cell survival, proliferation, and cell fate specification in the developing neural tube (Figure 3).



Extrinsic signals influence cellular proliferation, fate specification, and Figure 3. regionalization in the developing CNS. (A) Schematic of a transverse section through a typical vertebrate embryo showing the signaling centers that influence the patterning of the developing neural tube. Proliferating neural progenitors reside within the ventricular zone (VZ) adjacent to the central canal (CC). Post-mitotic, differentiated neurons reside in the mantle zone (MZ), located laterally to the VZ. Distinct neuronal subtypes are generated from defined progenitor domains located within the VZ along the dorsal-These progenitor domains are defined by the particular combination of ventral axis. transcription factors they express, which is influenced by opposing gradients of bone morphogenetic protein (BMP), wingless-INT (WNT), and sonic hedgehog (SHH) signals. Additionally, factors like WNTs can also influence regional identity along the rostrocaudal axis (Adapted from F. Ulloa et al. Cell Cycle 6(21):2640 (2007)). (B) Schematic of the regionalization of the developing neocortex. Signaling molecules such as

fibroblast growth factor 8 (FGF8), secreted from the anterior neural ridge and commissural plate; WNTs and BMPs, secreted from the cortical hem; and SHH, secreted from the medial ganglionic eminence, generate positional information during corticogenesis (Adapted from S-I. Ohnuma et al. *J. Biol. Chem.* 275(33):25358 (2000)). (C) Schematic of the posterior extension of the body axis of a Hamburger-Hamilton stage 10 (HH10) chick embryo representing the magnified area in the black box in the upper right hand corner. Stem zone specification and maintenance in the posterior of end of the developing neural tube involves FGF/ mitogen-activated protein kinase (MAPK)-mediated activity and presumably other putative signals (represented as 'X' in the schematic). Neuronal differentiation occurs when FGF/MAPK signaling is opposed by retinoic acid (RA) signaling eminating from the surrounding peraxial mesoderm (PM) (Adapted from M. Delfino-Machin et al. *Development* 132:4273 (2005)). D = dorsal; V = ventral; R = rostral; C = caudal; NC = notochord; RP = roofplate; FP = floorplate; DM = dermamyotome.

WNT signaling promotes proliferation of neural progenitors and influences cell specification by establishing gradients along both the rostro-caudal and dorsal-ventral axes of the neural tube (Kim et al. 2001; Nordstrom et al. 2002; Megason and McMahon, 2002). WNTs, specifically dorsal midline WNTs (WNT1 and WNT3a), through β-catenin/TCF signaling pathway induce proliferation of neural progenitors by promoting G1/S phase transition and by negatively regulating cell differentiation by inhibiting cell cycle exit, in part through the transcriptional up-regulation of D-type cyclins (Megason and McMahon, 2002). Mice lacking both WNT1 and WNT3a, which normally originate from the roof plate, display a dramatic reduction in dorsolateral neural progenitors within the neural tube (Ikeya et al. 1997). Ectopic expression of WNT1 in the mouse spinal cord increases the number of dividing progenitor cells (Dickinson et al. 1994), while the expression of a constitutively active β-catenin increases neural progenitor proliferation and decreases their rate of differentiation when ectopically expressed in mouse and chick spinal cords (Megason and McMahon, 2002; Ille et al. 2007). Bone morphogenetic

proteins (BMPs) are part of the transforming growth factor  $\beta$  (TGF $\beta$ ) family of secreted molecules that regulate proliferation of neural progenitors, through downstream Smad transcription factors (Massague, 1998), and possibly through the transcriptional activation of WNT genes (Chestnutt et al. 2004). The relationship between BMPs and WNTs is reciprocal, as WNTs can also up-regulate the expression of BMPs (Chestnutt et al. 2004; Ille et al. 2007). BMP signaling influences patterning along the dorsal-ventral axis of the neural tube by promoting the specification of cell types in dorsal regions (Wine-Lee et al. 2004). When activated, the BMP receptor BMPRIa positively influences the expression of the WNT1 gene (Panchision et al. 2001), which behaves as a mitogenic factor (Megason and McMahon, 2002). Inhibition of BMP signaling by the secreted polypeptide noggin blocks WNT1 and WNT3a expression in the roof plate and reduces proliferation of neural progenitors (Chestnutt et al. 2004). Transgenic mice that express constitutively active BMP receptors BMPRIa, which is not normally expressed in the early neural tube, increases the proliferation of neural progenitors, while expression of a constitutively active BMPRIb induces neuronal differentiation (Panchision et al. 2001). Since WNTs promote cell cycle progression, and BMPs promote cellular differentiation, it has been proposed that the cross-talk between BMP and WNT signaling pathways "fine-tunes" the growth and patterning of the neural tube (Ille et al. 2007).

During neurulation, BMPs are expressed at high levels at the lateral edges of the neural plate and later in the dorsal midline of the neural tube where they promote dorsal fates through a dorsal-to-ventral concentration gradient (Liem et al. 1995; Furuta et al. 1997). BMP signals originating from the dorsal midline oppose Sonic Hedgehog (SHH)

that promotes ventral fates by acting antagonistically from the notochord and ventral midline of the developing neural tube (Ericson et al. 1997; Briscoe and Ericson, 2001). The ventral-to-dorsal activation gradient of SHH is critical for generating the five neuronal progenitor domains in the ventral spinal cord (Jessell, 2000). SHH also behaves as a mitogenic and survival factor of neural progenitors in addition to its role in establishing specific progenitor domains within the developing ventral neural tube (Lai et al. 2003; Machold et al. 2003; Palma et al. 2004). SHH, by regulating downstream Gli transcription factor activity, promotes proliferation of neural progenitors cells in the cerebellum (Wechsler-Reya et al. 1999; Wallace, 1999; Dahmane et al. 1999; Pons et al. 2001), tectum, neocortex, and the spinal cord (Dahmane et al. 1999; Palma et al. 2004; Cayuso et al. 2006). Overexpression of Gli1, or a constitutively active Gli protein increases the proliferation of neural progenitors in the chick neural tube (Hynes et al. 1997; Cayuso et al. 2006). Conversely, the overexpression of a repressor form of Gli3, which inhibits the activation of SHH responsive genes, decreases cell proliferation and promotes cell death (Cayuso et al. 2006). Mice lacking SHH display defects in both cell proliferation and survival along the entire dorsal-ventral axis of the spinal cord (Borycki et al. 1999). Removal of the notochord and floor plate of the neural tube results in a apoptotic loss among neural progenitors, a phenomenon that can be rescued by providing an exogenous source of SHH, indicating that SHH is an important survival factor in the neural tube (Charrier et al. 2001).

#### **B.** Fibroblast Growth Factors and Neural Precursors

Neural precursor cells express FGF ligands and receptors from the primitive stages of morphogenesis (Ernfors et al., 1990; Gonzalez et al., 1990; Giordano et al., 1991; Powell et al., 1991; Nurcombe et al., 1993; Weise et al., 1993). Fibroblast growth factors (FGFs) are a large family of secreted proteins that includes twenty-two members in mammals (Dono, 2007). These signaling molecules activate a smaller family of membrane-bound receptors encoded in four genes (FGFR1-4) that, through alternative splicing, can produce numerous FGFR isoforms. FGFRs are single-pass transmembrane proteins with tyrosine kinase activity. Ligand binding to the extracellular domain of the receptor initiates a signaling pathway that ultimately results in changes in gene expression in the nucleus. The interaction between FGFs and their receptors also requires the intervention of heparin or heparin sulfate proteoglycans (HSPGs) that bind both the ligand and the receptor at specific domains and stabilize the formation of a receptor dimer bound to the FGF ligands (Kiefer et al., 1990). Thus the activity of FGFs is regulated at multiple levels, including growth factor expression, receptor binding affinity, and interaction with HSPG (Ornitz et al., 1996).

Only a handful of FGF molecules have roles in formation of the CNS, including neural induction (Streit et al., 2000), cell proliferation (Gensburger et al., 1987; Cattaneo and McKay, 1990; Kilpatrick and Bartlett, 1993; Ray & Gage, 1994; DeHamer et al., 1994; Johe et al., 1996; Vacarrino et al., 1999; Raballo et al., 2000), migration (Dono et al., 1998), and regionalization (Shimogori et al., 2004). FGF10 influences the differentiation of radial glial cells in the cortical VZ, which in turn affects corticogenesis

and patterning (Sahara and O'Leary, 2009). FGF8 plays a role in growth and patterning in the midbrain and anterior forebrain (Irving & Mason, 2000). FGF8 signaling also maintains a population of cells in the most caudal region of the primitive neural tube during spinal cord development (Novitch et al., 2003; Diez del Corral et al, 2004). In this region, known as the caudal stem zone, neural precursors are maintained in a proliferative, undifferentiated state, through the presence of FGF8 from the surrounding Caudal Hox gene expression in the stem zone depends on FGF/MAPK mesoderm. signaling (Delfino-Machin et al., 2005). As these undifferentiated cells migrate from the stem zone and enter the forming neural tube, they are exposed to a gradient of retinoic acid (RA) produced by the surrounding somites that opposes FGF8 signaling (Novitch et al., 2004; Diez del Corral et al., 2004). In this new environment, they acquire the competence to respond to dorsal-ventral (DV) patterning signals like SHH and to differentiate into neurons (Novitch et al., 2003; Diez del Corral et al., 2004). Blocking FGF activity also accelerates migration out of the stem zone into the forming neural tube (Mathis et al., 2001) (Figure 3 C). Consistent with these findings, overexpression of either a constitutively active mutated form of the FGF Receptor 1 (FGFR1) or recombinant FGF8 in the embryonic chick neural tube changes the transcriptional profile of effected cells, creating a rostral to caudal shift in the profile of Hox gene expression (Liu et al., 2001; Dasen et al., 2003). FGF15 indirectly influences proliferation in the cerebral cortex through its regulation of FGF8 in the anterior neural ridge in the developing forebrain (Borello et al., 2008).

FGF2 is required for expansion and maintenance of neural stem/progenitor cells both in vivo (Vacarrino et al., 1999; Raballo et al., 2000) and in vitro (Gensburger et al., 1987; Cattaneo & McKay, 1990; Ray, 1993; Kilpatrick and Bartlett, 1993; Ray & Gage, 1994; DeHamer et al., 1994; Johe et al., 1996). Intraventricular microinjection of recombinant FGF2 into the cerebral ventricles of rat embryos at the beginning of cortical neurogenesis increased both cortical volume and total number of neurons, while injection at the end of neurogenesis increased the number of cortical glia (Vaccarino et al., 1999). The authors also reported an increase in the percentage of proliferating neuroepethelial cells (Vaccarino et al., 1999). FGF2-deficient mice displayed defects in organization and differentiation of the embryonic cerebral cortex (Dono et al., 1998), and the number of cortical neurons and glia was reduced due to diminished expansion of the progenitor pool over the early period of neurogenesis (Vacarrino et al., 1999; Raballo et al., 2000). Recently, Wilcock et al. (2007) used live-imaging to monitor the cell cycle kinetics of single cells in organotypic sections of embryonic chick spinal cord exposed to FGF. Their findings indicated that the length of the cell cycle of neuroepithelial progenitors was accelerated after FGF treatment, exhibiting cell cycle kinetics more characteristic of earlier symmetrically dividing precursors relative to later asymmetrically dividing This in vivo finding supports earlier in vitro work showing FGF signaling precursors. expands the proliferation potential of neural precursor cells by increasing the number of cycles undergone before differentiating (DeHamer et al., 1994; Cavanagh et al., 1997; Lukaszewicz et al., 2002).

### C. Neural Stem Cells In Vitro

The relationship between growth factors and their influence on neural stem cell proliferation in vivo can be studied in a more controlled environment in vitro. Neural stem cells are harvested from a region of the embryonic brain or a region of the adult brain that has been demonstrated to contain dividing cells in vivo. The tissue is disaggregated and the dissociated cells are plated either as floating aggregates called neurospheres, or as a monolayer attached to the surface of a culture dish pre-treated with NSCs are then allowed to proliferate in supplemented medium in the fibronectin. presence of mitogens like FGF2 (Gensburger et al, 1987; Richards et al., 1992; Ray et al., 1993; Davis and Temple, 1994) or epidermal growth factor (EGF) (Reynolds and Weiss, 1992; Reynolds et al., 1992), which have been shown to influence the extent and rate of proliferation of neural stem and progenitor cell populations in vitro and in vivo (Reynolds and Weiss, 1992; Richards et al., 1992; Vescovi et al., 1993; Gage et al., 1995; Palmer et al., 1995, 1999; Kuhn et al., 1997; Shihabuddin et al., 1997; Wagner et al., 1999, Roy et al., 2000). NSCs also undergo changes in their responsiveness to mitogens over the course of embryogenesis, for example, embryonic FGF2-responsive NSCs later acquire EGF responsiveness (Reynolds et al., 1992; Johe et al., 1996; Tropepe et al., 1999) by a mechanism that involves up-regulation of the EGF receptor by FGF2 itself (Burrows et al., 1997; Lillien and Raphael, 2000). After some proliferation, the cells are either induced to differentiate by withdrawing the mitogens or by exposing the cells to instructive factor that induces some of the cells to develop into different lineages. In vitro, embryonic neural precursor cells can be directed toward particular cell fates using

different extracellular growth factors; however, the physiological relevance of these factors/conditions to the differentiation of neuroepithelial cells in vivo remains unclear. Both platelet-derived growth factor (PDGF) (Williams et al. 1997) and neurotrophin-3 (NT3) (Ghosh and Greenberg, 1995) have been shown to induce neuronal differentiation of neural precursor cells in vitro, while cytokines of the interleukin 6 (IL-6) subfamily leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), as well as cardiotrophin-1 (CT-1) promote differentiation into astrocytes (Johe et al. 1996; Bonni et al. 1997; Rajan and McKay, 1998; Nakashima et al. 1999). CNTF, LIF, and CT-1 activate the JAK/STAT pathway in neural precursors, and the inhibition of STAT3 disrupts astrocyte formation either in culture (Bonni et al. 1997; Rajan and McKay, 1998) or in vivo (Barnabe-Heider et al. 2005) in response to these factors. CT-1 is expressed in early cortical neurons and may promote the transition from neurogenesis to astrocytogenesis during CNS cerebral cortical development in vivo. This is accomplished through the formation of a feedback loop, where the antecedent neurons produce CT-1, which then influences subsequent cortical neuroepithelial cells to differentiate into astrocytes (Barnabe-Heider et al. 2005; Miller and Gauthier, 2007). The thyroid hormone T3 (Johe et al. 1996) and SHH (Alberta et al. 2001) are each capable of promoting oligodendrocyte differentiation in vitro. It is possible to follow lineage fates using immunocytochemistry by antibodies that recognize specific protein markers for astrocytes, oligodendrocytes, and neurons. The developmental potential of a single cell in culture can be accomplished through clonal analysis, in which cells are plated at low density and monitored to

determine if a single cell can give rise to the three major neural phenotypes (Reynolds and Weiss, 1996; Gritti et al., 1996; Johe et al., 1996; Kalyani et al., 1997).

#### V. REGULATION OF THE CELL CYCLE

#### A. Cyclins, Cyclin-dependent Kinases and Cyclin-dependent Kinase Inhibitors

Growth factors and morphogenetic molecules activate intracellular signaling pathways that stimulate the activity of cell cycle regulatory proteins necessary for cell cycle progression. The tightly regulated sequence of events known as the cell cycle are organized into 'phases': G1, S (DNA synthesis), G2 and M (Mitosis) (Figure 4). When cells are quiescent through serum deprivation, contact inhibition, or various anti-mitotic agents, they fail to make the G1 to S phase transition, instead entering a G0 phase. Progression through the cell cycle is dependent on the phase-specific expression of the cyclin-dependent kinases (CDKs; reviewed in Sherr and Roberts, 1995) and their regulatory subunits, the cyclins. The formation of cyclin-CDK complexes activates their kinase activity resulting in the subsequent phosphorylation of substrate proteins necessary for phase transition; for example, the association of D-type cyclins (cyclin D1, D2, or D3) and CDK4 or CDK6 results in the phosphorylation of cell cycle regulatory proteins essential for the G1 to S phase transition (Sherr and Roberts, 1999, 2004). E-type cyclins (cyclin E1 and E2) associate with CDK2 and is a key factor in the G1 checkpoint, promoting transition into S phase (Sherr and Roberts, 1999, 2004). Cyclin E/CDK-2 also plays a role in the initiation of DNA replication (Krude et al., 1997) and centrosome duplication (Mussman et al., 2000; Hinchcliffe et al., 1999). Cyclin E is normally induced at the transition from G1 into S phase and is rapidly degraded in early S phase (Pestell et

al., 1999; Landberg and Roos, 1997). A- and B-type cyclins together with their kinase partner CDK1 (CDC2) are the primary regulators of the G2/M-phase transition. Cyclin A (cyclin A1 or A2) also activates CDK2, and these various cyclin A-CDK complexes play roles in the S phase as well as in G2/M progression (Girard et al., 1991; Pagano et al., 1992). Cyclin A is up-regulated just prior to S phase and together with CDK-2 has been shown to phosphorylate proteins involved in DNA replication, such as CDC6 (Petersen et al., 1999; Coverley et al., 2000). During the G2/M transition, cyclin A-CDK1 activity is required for the initiation of prophase (Furuno et al., 1999). A number of studies have identified cyclin E and cyclin A as gene targets of the Rb/E2F pathway (see below). When cells exit mitosis, degradation of cyclin B is preceded by degradation of cyclin A and has been shown to be required for chromosomal alignment and progression to anaphase (Draetta et al., 1989; Minshull et al., 1990; den Elzen and Pines, 2001). B-type cyclins (cyclin B1, B2, and B3) associate with CDK1 (CDC2) and these cyclin B-CDK1 complexes operate during the M phase and regulate several steps necessary for successful cell division (Obaya and Sedivy, 2002).

Opposing the CDKs, are the CDK inhibitors (CKIs; reviewed in Sherr and Roberts, 1999). The CKIs have been categorized based on structure and for the CDKs they inhibit. The Ink4 (inhibitors of CDK4) class of CKIs specifically disrupts cyclin D-CDK4/6 activity. Members of the INK class of CKIs include p16<sup>INK4a</sup>, p15<sup>INK4b</sup>, p18<sup>INK4c</sup>, and p19<sup>INK4d</sup> proteins. The other class of CKIs is the CIP/KIPs, of which p21<sup>CIP1</sup>, p27<sup>KIP1</sup>, and p57<sup>KIP2</sup> are members. This second class of CKIs is less specific in its inhibition of CDKs, blocking the activities of cyclin E-, and A-CDKs, and p27<sup>Kip1</sup>

plays a role in inhibiting cyclin-CDK1 complexes (Toyoshima and Hunter, 1994; Nakayama et al., 2004; Pagano, 2004). Interstingly, although the Cip/Kip proteins are strong inhibitors of the cyclin E- and cyclin A-CDK2 complexes, p27<sup>Kip1</sup> is necessary for the assembly and stability of cyclin D-CDK4/6 complexes. There were no severe developmental defects seen in p27<sup>Kip1</sup> knockout mice, however mutant mice were significantly larger than control animals, suggesting mis-regulation of tissue growth (Fero et al., 1996; Kiyokawa et al., 1996; Nakayama et al., 1996).



**Figure 4.** A minimal model of the eukaryotic cell cycle and key cell cycle regulators. Progression of the cell cycle from G1 to S phase is positively regulated by the synthesis of D-type cyclins and their kinase partners cyclin-dependent kinases (CDKs), including CDK2 and CDK4. Mitogenic signals activate the pRb/E2F pathway (see below) to up-

regulate genes necessary for the G1 to S phase transition like cyclin E and A that together with CDK2 help push the cell into S phase. Cell cycle progression is negatively modulated by two families of CDK inhibitors (CDKIs), the INK4 and the CDK-inhibitory protein (CIP)/kinase-inhibitory protein (KIP) families (Sherr and Roberts, 1995). INK4 members include p16INK4a, p15INK4b, p18INK4c, and p19INK4d. The CIP/KIP family includes p21CIP1, p27KIP1, and p57KIP2. These molecules work by binding to and inactiving G1-cyclin/CDK complexes prohibiting the cell from transitioning to S phase and pushing the cell out of the cell cycle into G0 phase.

#### B. Regulation of the Cell Cycle by Growth Factors

The G1 to S phase transition is unique in that it is the only phase of the cell cycle that is regulated by growth factors (Figure 4). Entry into the cell cycle depends on mitogenic factors like FGF that activate downstream signaling pathways that stimulate the up-regulation of transcription of G1-type cyclins like D-type cyclins (cyclin D1, D2, D3) and facilitates the formation of cyclin-CDK complexes with either CDK4 or CDK6. Sequential cyclin D-CDK4/6 and cyclin E-CDK2 kinase activity results in hyperphosphorylation of the retinoblastoma (pRb) protein, disrupting its ability to bind and repress activator E2F proteins (see E2F Transcription Factors below). As a result, activator E2Fs bind to target promoters of cell cycle genes like cyclin E and cyclin A, promoting their transcription, and facilitating the G1 to S phase transition. This increase in cyclin E/A-CDK2 activity leads to the phosphorylation of the CKI p27<sup>Kip1</sup>, marking it for ubiquitin-mediated degradation by the proteasome (Pagano et al., 1995) and lowering the inhibitory threshold for transition from G1 to S phase.

## C. p27<sup>Kip1</sup> and Cell Cycle Withdrawal

In the context of CNS development and overall growth of an organism, understanding the mechanisms that dictate whether a cell becomes post-mitotic or
continues to proliferate is essential for understanding how cells generate specific cell lineages. Furthermore, mechanisms controlling withdrawal from the cell cycle will ultimately determine the number of post-mitotic neurons generated from neural progenitors. Cells made quiescent through contact inhibition or serum starvation, or cells exposed to anti-mitogenic signals like TGFß have shown to express elevated levels of p27<sup>Kip1</sup> protein (Polyak et al., 1994; Reynisdottir et al., 1995). Introducing mitogenic factors causes a rapid decrease in p27<sup>Kip1</sup> protein expression, through polyubiquitin-mediated degradation by the 26S proteasome (Pagano et al., 1995; Coats et al. 1996). The final cell division of a variety of precursor cells is correlated with changes in Cip/Kip expression levels, which suggests that CDK inhibitors play a role both in the exit of neural precursor cells from the cell cycle and in maintenance of the post-mitotic state.

The expression of p27<sup>Kip1</sup> is regulated mostly at the post-translational level (Hengst and Reed, 1996). The kinase activity of cyclin E/A-CDK2 phosphorylates p27<sup>Kip1</sup> at residue Thr-187, resulting in the proteasomal degradation of p27<sup>Kip1</sup> by the ubiquitin pathway (Pagano et al., 1995; Sheaff et al., 1997; Carrano et al., 1999; Sutterluty et al., 1999; Montagnoli et al., 1999; Tsvetkov et al., 1999). This pathway was demonstrated in transgenic mice expressing a mutated form of p27<sup>Kip1</sup> transgene that prevents its degradation by the proteasome (Malek et al., 2001). The authors reported only modest effects on proliferation both in vivo, however the in vitro analysis of cells derived from knockin mice showed that p27<sup>Kip1</sup> is degradaed through a mitogen-activated proteolytic pathway operating during the G1 phase (Malek et al., 2001). The Thr-187 phosphopeptide on p27<sup>Kip1</sup> is recognized by the Cks1b protein and is bound by both

Cks1b and the F-box protein Skp2 as part of the SCF<sup>Skp2</sup> E3 ubiquitin complex (Spruck et al., 2001; Ganoth et al., 2001; Hao et al., 2005).

### D. Ubiquitin-mediated Proteolysis and the SCF Ubiquitin Ligase Complex

Unidirectional movement through the cell cycle requires ubiquitin-mediated degradation of cell cycle regulators (Reed, 2003). Stringent control over ubiquitination pathways ensures that only the appropriate target proteins are recognized by the 26S proteasome. Proteasomes are large protein complexes are made up of a core 20S, which forms a central pore, and two 19S subunits (named according to their sedimentation coefficients). In eukaryotes, proteasomes can be found in both the nucleus and the cytoplasmic compartments. The proteasome is primarily responsible for degrading unnecessary or damaged proteins through a process called proteolysis, a chemical reaction involving proteases that break peptide bonds. Proteins are tagged for degradation through the covalent modification of a lysine residue in the form of polyubiquitinated chain by a tightly regulated multi-step pathway involving three ubiquitin-conjugating enzymes and ubiquitin protein ligases (Pollard et al., 2008).

In the first step leading to ubiquitination of a target substrate, an E1 ubiquitinactivating enzyme hydrolyzes ATP and adenylates a ubiquitin molecule. This activated ubiquitin molecule is then transferred to the active-site cysteine residue of E1 in concert with the adenylation of a second ubiquitin molecule. This adenylated ubiquitin is then transferred to the cysteine of a second enzyme, an E2 ubiquitin-conjugating enzyme. In the final step, an E3 ubiquitin ligase recognizes the appropriate target protein and catalyzes the transfer of ubiquitin from the E2 to the target substrate (Figure 5). A minimum of four ubiquitin monomers, a polyubiquitin chain, is required to mark a protein for proteasomal degradation (Pickart, 2001). Subunits of the proteasome cap complete the cycle by deubiquitinating the substrates as they are fed into the proteolytic central chamber. The released ubiquitin is then reutilized.

E3 ubiquitin ligases are responsible for targeting the correct protein substrate and for transferring the first ubiquitin molecule, therefore target specificity in the ubiquitination process is heavily dependent on the E3 family of enzymes. In the cell cycle of eukaryotes, E3 ubiquitin ligase complexes ensure phase transitions occur in succession and are properly timed, and the overlapping activity of the SCF and APC ligase complexes are essential for G1 to S phase transition (Figure 6).



Figure 5. Ubiquitin conjugation mechanism. Proteins are targeted for degradation by the proteasome by covalent modification of a lysine residue that requires the coordinated

reactions of three enzymes. In the first step, a ubiquitin-activating enzyme (known as E1) hydrolyzes ATP and adenylates a ubiquitin molecule. This is then transferred to E1's active-site cysteine residue in concert with the adenylation of a second ubiquitin. This adenylated ubiquitin is then transferred to a cysteine of a second enzyme, ubiquitin-conjugating enzyme (E2). In the last step, a member of a highly diverse class of enzymes known as ubiquitin ligases (E3) recognizes the specific protein to be ubiquitinated and catalyzes the transfer of ubiquitin from E2 to this target protein. After a protein has been ubiquitinated, it is recognized by the 19S regulatory particle, or "lid", in an ATP-dependent binding step. In general, chains of four or more ubiquitin molecules are sufficient for targeting to the proteasome. Subunits of the proteasome cap complete the cycle by de-ubiquitinating the substrate protein as it is fed into the proteolytic central chamber. The free ubiquitin is then recycled. Ub = ubiquitin, ADP = adenosine diphosphate, AMP = adenosine monophosphate, Pii = pyrophosphate. (Adapted from T. D. Pollard Cell Biology 2008, Saunders, 2nd Edition).





in regulation of cell cycle progression. At the metaphase-anaphase transition, the APC/C with associated Cdc20 specificity factor triggers the onset of anaphase by signaling the degradation of cyclin B. During mitosis, Cdh1 is phosphorylated by Cdk1-cyclin B making it incapable of binding APC/C, so that APC/C<sup>Cdh1</sup> activity is low. As Cdk1-cyclin B activity declines in anaphase, Cdh1 binds APC/C and Cdc20 dissociates from APC/C. After the onset of S phase, SCF (blown up to show the members of the complex and poly-ubiquitination of a representative target substrate, p27Kip1) together with the Skp2 and Cks1b specificity factors directs the degradation of phosphorylated p27Kip1 by ubiquitin-mediated proteasomal degradation. (Adapted from T. D. Pollard Cell Biology 2008, Saunders, 2nd Edition).

The SCF ubiquitin ligase complex consists of three core proteins in Skp1, Cul1, Rbx1, as well as an F-box protein that specifies the target substrate (Deshaies, 1999). Cull serves as a scaffold on which the Rbx1 and Skp1 subunits assemble. Rbx1 binds an E2 enzyme, while Skp1 binds the F-box protein subunit. F-box proteins make up the largest known class of E3 enzymes, thus the SCF complex is capable of ubiquitinating a multitude of substrates (Bai et al., 1996). Skp2, which is the F-box protein responsible for the recognition of p27<sup>Kip1</sup>, belongs to the Fbxl family that has at least 22 members in humans (Jin et al., 2004). Skp2 requires the CDC28 protein kinase regulatory subunit 1b (Cks1b) as a co-factor for specific recognition of p27Kip1 (Ganoth et al., 2001; Spruck et al., 2001; Hao et al., 2005). Cks1b belongs to a highly conserved family of essential cell cycle regulatory proteins that were originally identified through their ability to act as genetic suppressors of CDK mutations in yeast (Hadwiger et al., 1989; Hayles et al., There are two Cks proteins in mammals (Cks1 and Cks2; Richardson et al., 1986). 1990), but only Cks1b has the additional Skp2 binding activity that enables SCF to target p27<sup>Kip1</sup> specifically (Ganoth et al., 2001; Spruck et al., 2001). Converse to p27<sup>Kip1</sup> knockout mice, and consistent with the role of Cks1b in the destruction of p27Kip1, Cks1b

knockout mice are significantly smaller than wildtype and p27<sup>Kip1</sup> knockout animals and in vitro work showed a reduction in proliferation compared to wildtype (Spruck et al., 2001). The crystal structure of the Skp1-Skp2-Cks1b complex bound to the Thr-187 phosphopeptide on p27<sup>Kip1</sup> shows that Cks1b forms a major part of the binding surface that contacts phosphorylated p27<sup>Kip1</sup> and is capable of binding the leucine-rich repeat (LRR) domain and C-terminal tail of Skp2, while p27<sup>Kip1</sup> interacts with both Cks1b and Skp2 (Hao et al., 2005).

### E. APC/C Ubiquitin Ligase

Exit from mitosis is facilitated by the APC/C (anaphase-promoting complex/ cyclosome) primarily through the polyubiquitination and proteasomal degradation of cyclin B protein (Sudakin et al., 1995). An additional role for APC/C and its specificity factor Cdh1 (APC/C<sup>Cdh1</sup>) is to target Skp2 (Wei et al., 2004) and Cks1b (Bashir et al., 2004), the two specificity factors of the SCF<sup>Skp2</sup> complex. Thus, by destroying Skp2 and Cks1b in G1 phase, the APC/C<sup>Cdh1</sup> complex prevents the destruction of targets of the SCF<sup>Skp2</sup> complex, namely p27<sup>Kip1</sup>, ensuring that the cell spends the appropriate amount of time in G1 phase and does not commit to the G1-S phase transition prematurely (Bashir et al., 2004; Wei et al., 2004). The sequential, overlapping activity of the APC/C<sup>Cdh1</sup> and SCF<sup>Skp2</sup> degradation complexes creates an E3 ligase cycle that regulates the G1 to S phase transition and unidirectional progression through the cell cycle (Ang and Harper, 2004) (Figure 6).

# VI. E2F TRANSCRIPTION FACTORS: ACTIVATORS AND REPRESSORS OF TRANSCRIPTION

The retinoblastoma protein (pRb), along with pRb-related proteins p107 and p130 represent the major targets for cyclin D-CDK4/6 complexes, therefore, the pRb family members, also referred to as 'pocket proteins', are key regulators of cellular proliferation. Members of the E2F family of transcription factors positively and negatively regulate the expression of genes necessary for G1-S phase transition as well as many other genes necessary for cell cycle progression (Attwooll et al., 2004). The ability of E2Fs to regulate the transcription of E2F-responsive genes is dependent upon their interaction with pocket proteins, thus, the pRb/E2F pathway creates a direct link between extrinsic signals and gene transcription. In mammals, there are eight E2F gene products (E2F1– E2F8), where only two exist in flies and worms (Attwooll et al., 2004. Members of the E2F family of transcription factors are defined by a common conserved DNA-binding domain allows them to recognize a canonical TTTG/CG/CCGC consensus site (Zheng et al., 1999). Recognition of the consensus site by E2Fs 1-6 is dependent on heterodimerization with DP proteins (differentiation-regulated transcription factor-1 polypeptide; DP1 and DP2) (Helin et al., 1993), whereas E2F7 and E2F8 bind DNA as homodimers (Li et al., 2008).

E2Fs can be classified into two groups based on their ability to activate or repress target gene expression (Takahashi et al., 2000; Attwooll et al., 2004) (Figure 7 A). E2F1, E2F2 and E2F3a typically function as transcriptional activators, while E2F4, E2F5, or E2F3b are transcriptional repressors. E2F3a and E2F3b are different products of the E2f3 locus with distinct patterns of expression (Adams et al., 2000). E2F6 functions as a repressor, but independent of pocket proteins (Trimarchi et al., 2001). E2F7 and E2F8 lack a DP-binding domain but contain an E2F DNA-binding domain. As homodimers, E2F7 and E2F8 suppress the transcription of a subset of E2F-regulated targets (Li et al., 2008). E2Fs 1-5 are regulated by the pocket proteins pRb, p107, and p130 (Hurford et al., 1997) (Figure 7 B). While E2Fs 1-3a preferentially bind to pRb, the repressors E2F3b, E2F4 and E2F5 can bind to both p107 and p130; however, the dominant form of E2F in G0/G1 cells is the E2F4/p130 association (Takahashi et al., 2000) (Figure 7 B). E2F6-8 suppress transcription independently of pocket proteins (Trimarchi et al., 2001; Li et al., 2008) (Figure 7 B).



**Figure 7**. E2F family of transcription factors and pocket proteins. (A) The E2F family of winged-helix transcriptional regulators consists of eight known family members that can be distinguished based on their propensity to either activate or repress transcription of target genes. E2F members 1-5 have pocket protein binding domains that enable them to interact with members of the retinoblastoma protein (pRB) family that includes p107 (RBL1) and p130 (RBL2) (B) (Adapted from X. Xu et al. Genome Res. 17:1550 (2007)). (B) pRB primarily interacts with E2F activators, while p107 and p130 interact exclusively with E2F repressors E2F3b, E2F4, and E2F5. The E2F4/DP/p130 complex is the predominate E2F complex bound to cell cycle gene promoters in G0/G1

#### A. Classical Model for pRb/E2F Pathway

Expression of cell cycle genes is turned on or off through the reversible phasespecific recruitment of the E2F/DP pairs and associated chromatin remodeling enzymes (Blais and Dynlacht, 2007) (Figure 8). In quiescent or early G1 cells, E2F4/DP and associated pocket proteins (either p107 or p130) occupy promoters of targeted cell cycle genes, in some instances recruiting chromatin modifiers like the Sin3/HDAC complex, histone H3 methyltransferases (Suv39H) (Nicolas et al., 2000; Nielsen et al., 2000; van Oevelen, 2008) and DNA methyltransferase 1 (Robertson et al., 2000) (Figure 8 A). Unlike E2F1, E2F2, and E2F3, which are constitutively nuclear, E2F4 lacks a nuclear localization signal. Therefore, recruitment of E2F4 to selected promoters depends on its association with p107/p130, when these pocket proteins are hypophosphorylated, they enable E2F4 to localize to the nucleus. The presence of mitogenic factors stimulate G1 cyclin-CDK complexes that hyperphosphorylate pocket proteins, causing the release of activator E2Fs and freeing them to bind and activate target genes necessary for transition into S phase (Figure 8 B). When cells enter the cell cycle and proliferate, the repressor E2F4/DP/p130 complex is disrupted and/or replaced by the activator forms of E2F that promote gene expression. As cells progress into S phase, E2F4 and Sin3 dissociate from

repressed promoters, often replaced by activator E2F/DP dimers and increased histone H3 and H4 acetylation associated with the activation of gene expression (Blais and Dynlacht, 2007).



**Figure 8.** Regulation of cell cycle progression by the E2F/DP/pRb pathway. (A) In the absence of extrinsic growth signals (or in the presence of inhibitory signals), hypophosphorylated p130 binds cytoplasmic E2F4 allowing E2F4 to interact with the promoters of target genes. The E2F4/DP/p130 complex is thought to recruit histone deacetylases and repress genes required for the G1 to S phase transition. This blocks cell cycle progression at the restriction point late in G1. At the restriction point, cells assess external and internal stimuli and decide whether to commit to an additional round of DNA replication and division. (B) In the presence of extrinsic signaling, for example integrins and growth factors like FGF and EGF, the retinoblastoma protein (pRb) is hyperphosphorylated by cyclin-dependent kinases (cdks), which disrupts its sequestration of E2F activators, like E2F1, permitting the interaction between E2F1/DP/pRb and target gene promoters, allowing passage of the restriction point and cell cycle progression. Concordantly, hyperphosphorylated p130 is no longer able to bind E2F4, and the E2F4/DP/p130 complex dissociates from target promoters and becomes sequestered in the cytoplasm (Adapted from T. Pollard Cell Biology 2008 Saunders, 2nd Edition).

The ability of different activator/repressor E2Fs to regulate the same gene in response to mitogen activation of cell cycle progression makes E2F transcription factors a kind of "switch" mechanism, capable of turning cell cycle genes on and off. This makes the pRb/E2F pathway important for regulation of cell proliferation in response to growth factor stimulation. However, from an evolutionary perspective, the absence of the pRB/E2F pathway in yeast challenges their overall importance for cellular proliferation. E2Fs share a significant amount of functional redundancy, indicated by the lack of severe defects in knockout mice lacking single E2F proteins. The exception is E2F3a, which recent in vivo evidence suggests is the crucial activator for normal rodent development, superseding the other activator E2Fs (Tsai et al., 2008). Only when all three activator E2Fs, E2F1, E2F2, and E2F3a, are lost simultaneously in mouse embryonic fibroblasts is there a complete block in proliferation (Wu et al., 2001). Combined inactivation of E2F4 and E2F5 results in embryonic death, indicating a compensatory relationship between the two in vivo (Gaubatz et al., 2000). However, the functional redundancy

among mammalian E2Fs may benefit a more complex multicellular organism, as opposed to less complex eukaryotes like the fly and worm, and even less complex, unicellular organisms like the yeast, which have none, so that the complexity of the pRB/E2F pathway reflects the complexity of the organism.

During neurogenesis neural precursor cells receive a host of instructive extinsic signals that either promote their proliferation or coax them to commit to a particular neural cell lineage contributing ultimately to the patterning of the CNS. Up to now, the mechanisms underlying the decision made by NPCs during early neurogenesis to either continue proliferating or withdraw from the cell cycle and commit to a particular neural cell lineage fate, remain either elusive or unclearly/only loosely defined. In the forthcoming pages, we describe a mechanism whereby NPCs prolong their proliferative life by degrading the CKI p27KIP1 protein in response to FGF signaling, specifically FGF2. Our results show that this is accomplished in part by up-regulating Cks1b, a key member of the SCF<sup>Skp2</sup> ubiquitin-ligase complex that targets p27<sup>KIP1</sup> for ubiquitinmediated proteolysis, through E2F transcription factors and a CDE-CHR consensus element located in the regulatory sequence immediately upstream of the Cks1b transcription start site. These new findings present a more dynamic picture of how mitogenic factors promote cell cycle progression in NPCs during early neurogenesis by degrading key CDK inhibitors in addition to promoting the activation cell cycle promoting genes like G1-cyclin/CDKs.

## **Chapter II**

#### RESULTS

Neurogenesis involves the proliferation and differentiation of precursor cells leading to the populating and patterning of the brain and spinal cord. As neural precursor cells exit the cell cycle they commit to neuronal or glial lineages. Whether cell cycle exit and cyto-differentiation are isolated incidents or inseparable events remains an open question. A number of cell cycle regulatory molecules have additional functional roles in regulating individual steps in neurogenesis, including cell cycle exit (Ohnuma and Harris, 2003), cell migration (Bielas et al. 2004), and neuronal differentiation (Bertrand et al. 2002). Existing evidence has shown that Cip/Kip CKIs p21<sup>Cip1</sup>, p27<sup>Kip1</sup>, and p57<sup>Kip2</sup> have additional roles in development of the CNS independent of cell cycle regulation. p27<sup>Kip1</sup> in particular has been implicated in neuronal differentiation and migration in the developing rodent cortex (Nguyen et al. 2006), as well as glial fate specification in the retina (Durand et al. 1997), independent of its role in promoting cell cycle arrest of neural progenitors during embryogenesis (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996).

# p27<sup>Kip1</sup> protein is expressed by post-mitotic neurons and is largely absent from the ventricular zone during neurogenesis

To examine the functional role of  $p27^{Kip1}$  in neural precursor cell cycle exit and differentiation, we looked at the expression pattern of  $p27^{Kip1}$  protein in the developing CNS during the period coinciding with the onset of neurogenesis. We observed that  $p27^{Kip1}$  was expressed mostly in post-mitotic neurons residing in the mantle zone (MZ) of

the developing spinal cord (Figure 9), and that this expression pattern was conserved among vertebrates, from chicken to rat to human.  $p27^{Kip1}$  protein expression coincided with expression of the neuronal marker neuronal tubulin type-III ß (N-tubulin, or Tuj1) (Figures 9, 10 A and 11 A) but was mostly absent from actively dividing ventricular zone cells (Figure 9). The subcellular localization of  $p27^{Kip1}$  in the developing spinal cord appeared to be dependent upon both the developmental stage as well as the axial level. At E12,  $p27^{Kip1}$  was mostly nuclear in both the dorsal (Figure 10 B-E) and ventral (Figure 10 E-G) aspects regardless of axial level, whereas in the E14.5 rat spinal cord,  $p27^{Kip1}$  was cytoplasmic in the more dorsal-medial aspect (Figure 11 B-D), a mixture of cytoplasmic and nuclear in the more ventral-medial aspect (Figure 11 H-J) at cervical levels. Throughout the period coinciding with neurogenesis in rat,  $p27^{Kip1}$  was consistently present in Tuj1-positive axons in the ventral root (data not shown).



**Figure 9.** The expression of  $p27^{Kip1}$  protein is conserved in the developing vertebrate CNS. Spinal cords from embryonic day 4.5 (E4.5) chick (A), E14.5 rat (B), and 7-week-old human fetus (C) were co-immunostained with the  $p27^{Kip1}$  antibody (green; A-C) and neuronal class III beta-tubulin (Tuj1; red) to show postmitotic neurons and their fibers. The white bars in (A), (B), and (C) each represent 100 µm.



**Figure 10.** In E12 rat spinal cord, the subcellular localization of  $p27^{Kip1}$  protein is predominately nuclear. Shown in (A) is a confocal image of a cross-section through an E12 rat spinal cord. Antibodies against  $p27^{Kip1}$  (red), neuronal tubulin type III (Tuj1, green), and DAPI (blue) were used to demarcate p27 protein expression, post-mitotic neurons, and nuclei, respectively. The white boxes in (A) represent the location of the magnified confocal images shown in B-G.  $p27^{Kip1}$  subcellular localization was mostly nuclear in both the dorsal (B-D) and ventral (E-G) aspects of the developing spinal cord. In the ventral horn,  $p27^{Kip1}$  was mostly nuclear in post-mitotic motor neurons in the ventral horn (E-G). The white bars in (A) and (G) represent 100 µm and 20 µm, respectively. VZ = ventricular zone, RF = roof plate, DRG = dorsal root ganglia, MN = motor neurons, FP = floor plate.



**Figure 11**. In E14.5 rat spinal cord, the subcellular localization of  $p27^{Kip1}$  protein varies depending on the axial level of the spinal cord. Shown in (A) is a confocal image of a cross-section through an E14.5 rat spinal cord at the cervical level. Antibodies against  $p27^{Kip1}$  (red), neuronal tubulin type III (Tuj1, green), and DAPI were used to demarcate p27 protein expression, post-mitotic neurons, and nuclei, respectively. The white boxes in (A) represent the location of the magnified confocal images shown in B-J.  $p27^{Kip1}$  subcellular localization was mostly cytoplasmic in the dorsal-medial section of the developing spinal cord (B-D); however,  $p27^{Kip1}$  appeared to be both nuclear as well as cytoplasmic in more ventral-medial sections (E-G). In the ventral horn,  $p27^{Kip1}$  was mostly nuclear in post-mitotic motor neurons (H-J). The white bars in (A) and (J) represent 100 and 20 µm, respectively. VZ = ventricular zone, RF = roof plate, DRG = dorsal root ganglia, MZ = mantle zone, MN = motor neurons, FP = floor plate.

In E12 rat cerebral cortex, p27<sup>Kip1</sup> protein expression coincided with the neuronal markers Tuj1 (Figure 12 A-D) and HuD (Figure 12 E-H) in the outer cortical plate. A small fraction of p27Kip1-positive cells were detected in the ventricular and intermediate zones, however, these cells usually co-expressed lineage-specific markers like Tuj1 (Figure 12 B-D) and HuD (Figure 12 F-H). During mid- to late-neurogenesis, p27<sup>Kip1</sup>immunoreactivity did not coincide with phospho-histone H3 (pH3)-positive proliferating cells in the VZ and SVZ layers at either E14.5 (Figure 13 B-D) or E16 (Figure 14 J-L), but did coincide with Tbr1-positive early-born cerebral cortical neurons (Figure 13 H-J; Figure 14 G-I) and HuD- (Figure 13 K-M) and Tuj1-positive (Figure 14 D-F) neurons. Again, a small fraction of p27Kip1-positive cells at the VZ/SVZ boundary, but these cells often expressed markers for differentiated cell types like IB4 (Figure 13 E-G; Figure 14 A-C), an endothelial marker that labels blood vessels, Tbr-1, or HuD and Tuj1, indicated that these p27<sup>Kip1</sup>-positive cells have committed to a particular lineage and are in the process of migrating out of the VZ. Finally, we also documented the expression pattern of p27<sup>Kip1</sup> in the retina during retinal neurogenesis. Consistent with what we observed in the developing CNS, p27<sup>Kip1</sup> expression coincided with Tuj1-positive neuronal cells residing in the differentiated cell layer (DCL) (Figure 15 A-C) but not with cells in the neuroblast cell layer (NCL) in the developing retinal neuroepithelium. p27<sup>Kip1</sup> is also expressed in the lens, a compartment mostly made up of non-neuronal cells that were positive for IB4 (Figure 15 D-F), and is mostly absent from proliferating cells marked by phospho-histone H3 (Figure 15 G-I), consistent with the expression pattern seen for p27<sup>Kip1</sup> in the developing brain and spinal cord.



Figure 12. p27<sup>Kip1</sup> is expressed mostly in neuronal populations in the developing cerebral cortex in E12 rat brain. Shown in (A) is a micrograph of a sagittal section through an E12 rat brain taken at 4x magnification labeled with antibodies against Tuj1 (A-D) and HuD (E-H), p27<sup>Kip1</sup>, and DAPI to label all nuclei. The white boxes in (A) and (E) demarcate the region of the cerebral cortical shown in (B-D) and (F-H), respectively, at 20x magnification. Each staining is from the same embryo but at different positions along the lateral-medial axis of the brain, with (B-D) being more medial and (K-M) being more lateral. p27Kip1 is expressed mostly in Tuj1- and HuD-positive post-mitotic neurons, however, a small fraction of p27Kip1-positive cells were seen in the ventricular and intermediate zones. These cells often co-express lineage specific markers like Tuj1 (B-D) and HuD (F-H), as indicated by the white arrows. This suggests that p27<sup>Kip1</sup>positive cells are most likely differentiated neurons that have exited the cell cycle and are in the process of migrating away from the ventricular zone. The white bar in (E) and (H) represents 100  $\mu$ m. V = ventricle, BG = basal ganglia, H = hippocampus, DT = dorsal telencephalon, VT = ventral telencephalon, VZ = ventricular zone, IZ = intermediate zone, and CP = cortical plate.



**Figure 13.**  $p27^{Kip1}$  is expressed mostly in neuronal populations in the developing cerebral cortex in E14.5 rat brain. Shown in (A) is a micrograph of a coronal section through an E14.5 rat brain labeled with antibodies against HuD, a neuronal marker,  $p27^{Kip1}$ , and DAPI to label all nuclei. The white box in (A) demarcates the region of the cerebral cortical shown in (B-M) at higher magnification. Each staining is from the same embryo but at different positions along the anterior-posterior axis of the brain, with (B-D) being most posterior and (K-M) being most anterior. In the cerebral cortex, cells expressing phosphorylated histone 3 (pH3), a marker for proliferative cells, did not co-express  $p27^{Kip1}$  (B-D). Most of the cells in the ventricular zone that expressed  $p27^{Kip1}$  were also positive for IB4, an endothelial marker that labels blood vessels (E-G).  $p27^{Kip1}$  was expressed mostly in post-mitotic neurons, as indicated by co-expression of neuronal markers Tbr1 (H-J) and HuD (K-M). The white bar in (A) and (M) represents 100 µm. VZ = ventricular zone, IZ = intermediate zone, and CP = cortical plate.



**Figure 14**. p27<sup>Kip1</sup> is expressed mostly in neuronal populations in the developing cerebral cortex in E16.5 rat brain. Images (A-L) show high magnification confocal images taken of coronal sections through the cerebral cortex of E16.5 rat brain. Each staining is from

the same embryo but at different positions along the anterior-posterior axis of the brain, with (A-C) being most posterior and (K-M) being most anterior. In the cerebral cortex, most of the cells in the ventricular zone/subventricular zone that expressed  $p27^{Kip1}$  were also positive for IB4, an endothelial marker that labels blood vessels (A-C). Examples of  $p27^{Kip1}$ -positive cells that are also positive for IB4 are demarcated by white asterisks in (A-C).  $p27^{Kip1}$  was expressed mostly in mostly in post-mitotic neurons, as indicated by Tuj1 (D-F) and Tbr1 (G-I) immunoreactivity. A prominent example of a cell in the ventricular zone co-expressing  $p27^{Kip1}$ Kip1 and Tuj1 is demarcated by a white arrow in (D-F). Ventricular/subventricular zone cells expressing phosphorylated histone 3 (pH3), a marker for proliferative cells, did not co-express  $p27^{Kip1}$  (J-L). The white bar in (L) represents 100 µm. VZ = ventricular zone, SVZ = subventricular zone, IZ = intermediate zone, and CP = cortical plate.



**Figure 15**.  $p27^{Kip1}$  expression pattern in the developing retina of E14.5 rat. Shown are immunostainings of cross-sections through retinas from E14.5 rat taken at 20x magnification. Expression of  $p27^{Kip1}$  protein was observed in both the differentiated cell layer (DCL), identified by Tuj1 immunoreactivity (A-C), and lens, which consists mostly of non-neuronal cells, at a developmental stage coinciding with the onset of retinal neurogenesis. We also observed  $p27^{Kip1}$  immunoreactivity in the lens compartment that was also positive for the endothelial marker IB4 (D-F), but  $p27^{Kip1}$  was not expressed by proliferating cells indicated by pH3 immunoreactivity (G-I). The white bar in (C) represents 100 µm. NBL = neuroblast layer, DCL = differentiated cell layer, L = lens.

p27<sup>Kip1</sup> mRNA is found in both neurons and neural progenitors in the VZ, while mRNA for Cks1b and Skp2 are found mainly in the VZ

In contrast to the expression pattern of p27Kip1 protein, the message for p27Kip1 was seen throughout the developing spinal cord (Figure 16) and cerebral cortex (Figure 17) with higher expression levels in the VZ. As controls, we designed probes for Gap43 (Figure 16 B,E; Figure 17 A,E) and neurogenin2 (Ngn2) (Figure 16 C,F) to demarcate the mantle layer and ventricular zone, respectively. p27Kip1 mRNA was detected in the mantle layer (ML) and cortical plate regions of the spinal cord (Figure 16 G,J) and brain (Figure 17 B,F), respectively, as well as in the VZ. Our observation that p27Kip1 transcripts were expressed in both the proliferative zone and post-mitotic cells in spinal cord and cerebral cortex suggests that the expression of p27<sup>Kip1</sup> protein is regulated at the post-transcriptional level. Existing evidence suggests that translational (Kullmann et al., 2002) and post-translational (Tsvetkov et al., 1999; Zhang et al., 2005) events are largely responsible for control of p27Kip1 protein expression. Perhaps the most wellcharacterized mechanism for regulation of p27Kip1 protein is through ubiquitination. Ubiquitination and eventual proteasomal degradation of p27<sup>Kip1</sup> requires its phosphorylation at Thr187 and subsequent recognition by CDC28 protein kinase regulatory subunit 1B (Cks1b) that binds to Skp2, a member of the F-box family of proteins that associates with Skp1, Cul-1 and ROC1/Rbx1 to form the SCF ubiquitin ligase complex. Cks1b is a small adaptor protein that binds Skp2 and greatly increases binding of p27<sup>Kip1</sup> to Skp2 (Ganoth et al., 2001). To investigate whether the SCF<sup>Skp2</sup> components Cks1b and Skp2 play any role in the ubiquitin-mediated degradation of p27Kip1 in the VZ during CNS development, we looked at their expression patterns at the

same developmental stages in mouse. We detected Cks1b (Figure 16 H,K; Figure 17 C,G) and Skp2 (Figure 16 I,L; Figure 17 D,H) mostly in the VZ in both the spinal cord and cerebral cortex, with reduced levels restricted to the thin lining of ependymal cells surrounding the central canal at later stages in the spinal cord. The VZ is an area known to contain actively dividing neural precursor cells that do not express the p27<sup>Kip1</sup> protein. Based on these observations, we hypothesized that neural progenitors use SCF<sup>Skp2</sup> ubiquitin-mediated proteolysis as a mechanism to suppress the expression of p27<sup>Kip1</sup> protein as a means to preserve their proliferative state during early neurogenesis.



**Figure 16.** p27<sup>Kip1</sup>, Cks1b, and Skp2 transcripts in the developing neural tube. In situ hybridization data using transverse sections from embryonic day (E)12.5 (A-C,G-I) and E14.5 (D-F,J-L) mouse spinal cord. Detection of hematoxylin and eosin (A,D), Gap43, which labels post-mitotic neurons (B,E), and Neurogenin-2, which labels cells residing in the ventricular zone (C,F). (G,J) Unlike p27<sup>Kip1</sup> protein expression, the mRNA for p27<sup>Kip1</sup> was found both in the mantle layer (ML) and ventricular zone (VZ) of the developing neural tube. (H,K) Cks1b and Skp2 (I,L), two components of the SCF<sup>Skp2</sup> E3 ligase complex, were detected exclusively within the VZ. Sense probes served as negative controls. Black bars in micrographs (A) and (D) represent 100  $\mu$ m. Landmarks



**Figure 17.** p27<sup>Kip1</sup>, Cks1b, and Skp2 transcripts in the developing cerebral cortex. In situ hybridization data using transverse sections from E14.5 mouse whole brain (A-D) and cortex (E-H). Gap43 hybridization demarcated post-mitotic neuronal populations (A,E). (B,F) p27<sup>Kip1</sup> hybridization labeled cells in the ventricular zone (VZ), intermediate zone (IZ), and cortical plate (CP). Transcripts for Cks1b (C,G) and Skp2 (D,H), two components of the SCF<sup>Skp2</sup> E3 ligase complex, were detected in the VZ. No signal was detected when a sense probe was used. Black box in micrograph (A) denotes cortical region shown in (E-H). Black bars in micrographs (A) and (E) represents 100 µm. Landmarks in (E) are: VZ = ventricular zone; IZ = intermediate zone; CP = cortical plate; MGE = medial ganglionic eminence; LGE = lateral ganglionic eminence.

## FGF2 regulates Cks1b but not p27<sup>Kip1</sup> at the transcriptional level

In order to test this hypothesis at the cellular level, we isolated neural progenitor cells from E14 rat cortex and studied their proliferation under different conditions of mitogen stimulation. Isolated neural progenitor cells were passaged at least once (P1) after the initial harvest (P0) and were maintained in basic fibroblast growth factor (FGF2). FGF2 is a mitogenic factor used for in vitro studies of neural progenitors and is also required for expansion and long-term survival of neural progenitor cells at least once is to eliminate neurons and non neural cells that are carried over from dissociation of the nervous tissue. The panel in Figure 10 shows that at P0 (harvest) cultures consist of mostly of progenitor cells as evidenced by staining with nestin and a smaller population of neurons (Figure 18 B,C,D,F), and non neural cells (data not shown), and neural progenitors (Figure 18 E,F). Consistent with in vivo expression pattern,  $p27^{Kip1}$  expression is seen only in post-mitotic neurons (Figure 18 A -C).


**Figure 18**.  $p27^{Kip1}$  is expressed in post-mitotic neurons at the time of neural precursor cell isolation. Neural progenitors were harvested from E14 rat telencephalon and grown as monolayers in culture. At the time of initial plating, termed passage 0 (P0), these cultures contain a mixture of neural cells, including neural precursors, neurons, and neuroglia. Shown are micrographs illustrating that at P0, neurons labeled by Tuj1 (B,E) carried over from harvest express  $p27^{Kip1}$  (A,C), while the majority of nestin-positive cells (D,F), a marker for neural stem cells, do not express  $p27^{Kip1}$ . White arrows in (A-C) demarcate neural cells positive for both  $p27^{Kip1}$  (A,C) and Tuj1 (B,C). White bar in bottom right corner of micrograph (F) represents 50 µm.

To further test whether p27Kip1 mRNA expression is mimicking the pattern of expression in vivo we analyzed by RT-PCR proliferating and non-proliferating progenitor cells by RT-PCR. cDNA was made from parallel cultures of proliferating and nondividing P1 neural progenitors cells in the presence or absence of FGF2, respectively, and used in RT-PCR analysis. We found that p27Kip1 mRNA was present in both FGFdeprived neural progenitor cells and progenitors that were stimulated with FGF2 for 1, 6, and 24 hours (Figure 19 A). As a positive control, we looked at the level of cyclophilin A transcripts from the same cDNA samples. We also investigated the expression of p27<sup>Kip1</sup> protein in neural progenitors in vitro using immuncytochemistry. Parallel cultures of P1 neural progenitor cells were either maintained in FGF2 to promote their proliferation (Figure 19 B,C), or deprived of FGF2 for 4 days (Figure 19 D,E) to slow their rate of cell division. p27Kip1 was detected only in the neural progenitor cultures that had been deprived of FGF2 (Figure 19 D). These data were quantified by clonal analysis and p27<sup>Kip1</sup>-positive cells were analyzed as a ratio of total cells within a colony (Figure 19 F). Our clonal experiments show that p27<sup>Kip1</sup>-positive cells constitute less than one percent of the entire population of proliferating neural progenitor cells, whereas approximately 76% were positive when deprived of FGF2 for 4 days. Our in vitro

findings, that p27<sup>Kip1</sup> mRNA is found in both proliferating and non-proliferating cells and that in contrast, p27<sup>Kip1</sup> protein is only seen in non-dividing cells, is consistent with the idea that that p27<sup>Kip1</sup> is regulated at the post-transcriptional level in neural progenitor cells.



**Figure 19.** p27<sup>Kip1</sup> expression in NPCs in vitro in response to FGF2. Neural progenitors were harvested from the cortex of E14 rat pups and grown as monolayers on dishes coated with fibronectin and maintained in N2 media containing FGF2 (10ng/ml). For experiments involving various treatments with FGF2, culture conditions were manipulated as indicated. (A) cDNA was made from NPCs exposed to FGF2 at the time points indicated and RT-PCR was used to detect p27<sup>Kip1</sup> mRNA levels . (B-E) Immunocytochemical analysis of p27<sup>Kip1</sup> protein expression in NPC cultures in the presence (B,C) and absence (D,E) of FGF2. (F) Quantitation of immunocytochemical data shown in (B-E). The number of p27<sup>Kip1</sup>-positive cells was counted in proportion to

the total number of cells within the indicated number of colonies (F). White bar in bottom right corner of micrograph (E) represents 50  $\mu$ m.

We then used this in vitro system to analyze the regulation of the SCF<sup>Skp2</sup> ligase complex in progenitor cells. We chose to focus on the regulation of Cks1b, as Cks1b is the crucial adaptor protein of the SCF<sup>Skp2</sup> complex necessary for recognizing p27Kip1 specifically as a target for degradation. Furthermore, expression data for Cks1b during embryonic development in CNS showed that mRNA is found mainly within the VZ, reinforcing the idea that the regulation of Cks1b expression is crucial for p27Kip1 degradation in neural progenitor cells. Once again, parallel cultures of P1 neural progenitors were deprived or stimulated with FGF2 to promote their proliferation and quiescence, respectively. Our RT-PCR revealed that levels of Cks1b transcript were reduced in neural progenitors deprived of FGF2 relative to FGF-simulated cultures (Figure 20 A). We next analyzed by western blot the expression of Cks1b protein using lysates from cultures subjected to different conditions of FGF2. As a positive control, lysates made from cells ectopically expressing recombinant Cks1b were used. Levels of Gapdh were monitored for equal loading. The level of expression of Cks1b protein varied considerably, depending on whether cultures were exposed to FGF2 or not. In the absence of FGF2, the expression of Cks1b was greatly reduced after 2 days, but could be recovered after exposure to FGF2 for 24 hours (Figure 20 B). These results indicate that Cks1b is regulated by FGF2 at the transcriptional level in neural progenitor cells. Further, these findings are similar to what we observed in the developing CNS, that Cks1b mRNAs are expressed mostly within the proliferative layer (VZ).



**Figure 20.** FGF controls Cks1b expression in NPC in vitro. Neural progenitors were harvested from the cortex of E14 rat pups and grown as monolayers on dishes coated with fibronectin and maintained in N2 media containing FGF2 (10ng/ml). For experiments involving various treatments with FGF2, culture conditions were manipulated as indicated. (A) cDNA was made from NPCs exposed to FGF2 at the indicated time points and RT-PCR was used to detect Cks1b mRNA levels. (B) Western blot analysis showing the fluctuation in Cks1b protein expression in NPC cultures in response to FGF2 deprivation and re-application for the indicated days. (C-H) Immunocytochemical analysis of p27<sup>Kip1</sup> protein expression in NPC cultures in the presence of FGF2 (C,F), in FGF2-deprived cultures (D,G), and after the re-application of FGF2 (E,H) for the indicated durations. MCM2, a marker for cells in G1 and S-phase, was used as a counter-stain. White bar in bottom right corner of micrograph (H) represents 50 μm.

To see whether there is an inverse relationship between the changes of Cks1b

expression and p27Kip1 we also looked by immunocytochemistry at the levels of p27Kip1

protein in neural progenitor cultures subjected to similar treatment as in Figure 19 B (Figure 20 C-H). As a counter-stain, we used a marker for proliferating cells, MCM2, which is expressed by actively dividing cells entering S phase (Figure 20 F-H). We found that levels of p27<sup>Kip1</sup> protein expression were virtually undetectable in proliferating cultures (Figure 20 C,F), but increased dramatically in response to withdrawal of the mitogenic factor (Figure 20 D,G). Subsequent re-application of FGF2 led to the reversal in p27Kip1 expression (Figure 20 E,H). As we predicted, expression of MCM2 and p27Kip1 had an inverse relationship with respect to the presence and absence of FGF2 on dividing and non-dividing neural progenitors, respectively. Our in vitro data show that neural precursors harvested from E14 rat cortex and maintained in the presence of FGF2 express high levels of Cks1b protein and undetectable levels of p27<sup>Kip1</sup> protein, while removal of FGF2 caused a sharp decrease in Cks1b protein levels and corresponding increase in p27Kip1 protein. Furthermore, our RT-PCR data indicated that Cks1b mRNA is reduced in non-dividing neural precursors but is present in proliferating precursor cells, suggesting to us that Cks1b is being regulated at the level of transcription. Based on these observations, we hypothesized that FGF2 signaling regulates the expression of Cks1b in neural progenitors, which prevents the expression of p27Kip1 and promotes proliferation (Figure 21).



**Figure 21.** Central hypothesis. Based on our preliminary data, we have proposed the above minimal model for how FGF2 drives Cks1b expression in proliferating NPCs. Based on our observations, we hypothesize that FGF2 signaling regulates the expression of Cks1b in neural progenitors, which prevents the expression of p27<sup>Kip1</sup> and promotes proliferation.

Our data indicated that Cks1b is regulated by FGF2 at the transcriptional level in neural progenitor cells *in vitro*. To gain insight into the regulatory program(s) involved in the transcription of Cks1b, we undertook several experiments that want to draw a causal relationship between FGF2 and Cks1b transcription. The following experimental outline was followed to achieve this goal.

- The regulatory elements that control Cks1b transcription probably are conserved in vertebrates because the pattern of expression of p27<sup>Kip1</sup> is conserved from chick to Human. Therefore genomic analysis of the promoter region and other intervening sequences followed by in silico analysis of the promoter region will identify transcription factors that might be involved in the expression of Cks1b.
- 2) Determine whether the putative transcription factors are expressed in neural precursor cells.
- Perform ChIP assays to identify transcription factors that bind the promoter region of Cks1b.
- 4) Promoter analysis using luciferase assays in neural progenitors and other cells to identify the promoter region of Cks1b that is regulated by FGF2.

5) Mutagenesis of essential transcription consensus sequences followed by luciferase assays to draw a causal relationship between the putative transcription factors and regulation of Cks1b expression by FGF2.

We first analyzed the sequence conservation of the human Cks1b gene among the genomes of different vertebrate species using the UCSC genome web browser (Figure 22 A). The loci of the Cks1b and Shc1 genes are indicated, and the blue histogram below represents the level of conservation among the vertebrate species listed for the overlying sequence (Figure 22 A). Next, we performed a sequence alignment of the promoters for the human, rat, mouse, and dog Cks1b genes. Using the web-based alignment tool ClustalW, we analyzed a 1 kb sequence upstream of the putative transcription start site for regions of high conservation. This sequence was also scanned using the TRANSFAC and Genomatix databases in an attempt to identify conserved consensus transcription factor binding sites (TFBS) (Figure 22 B,C). Nucleotide sequences recognized by DNAbinding proteins form more or less a loose consensus, which limits the accuracy of sequence-based prediction of transcription factor binding sites. However, algorithms that quantitatively compare promoter sequences to documented consensus binding motifs are nonetheless useful for generating testable hypotheses. We identified multiple conserved TFBS that could potentially be involved in the regulation of Cks1b transcription (Figure 22 C). Among them were the consensus for E2F proteins that are a family of activator and repressor transcription factors important in controlling the expression of genes important for DNA replication as well as further cell cycle progression. The cell cycle homology region (CHR) and the cell cycle-dependent element (CDE) consensus sequences, downstream of and in close proximity to the E2F consensus site, are repressor elements common in promoters of cell cycle genes. We also identified a consensus site

for Smad transcriptional factors that are downstream effectors of TGF- $\beta$  signaling and are predominately transcriptional repressors. Additional potential activator sites included several CAAT box activator sites, including a NF-Y binding site, and consensus sites for FoxMI and Elk-1, an ETS transcription factor downstream of mitogen-activated MAPKinase signaling. We focused our attention on the E2F consensus site, because of the importance and well-established roles of E2Fs in controlling cell cycle regulated genes and because of their ability to mediate extracellular signals and cell cycle progression or arrest. Taken together, our in vitro findings coupled with our findings from in silico promoter analyses led us to hypothesize that mitogen signaling regulates the expression of Cks1b at the transcriptional level, possibly through the interaction between E2Fs and the promoter region of Cks1b, in proliferating and non-dividing neural progenitors as a mechanism for controlling the levels of p27<sup>KIP1</sup> expression.



_					~
SP1	E2F	NFY	ETS1	FOXM1	Activators
000000000000000000000000000000000000000	TGTCCCGC	CCAATAGGAAGT	GGAAGT	TGTTTGAA	Sequence
-459/-451	-124/-116	-121/-110	-115/-110	-99-/92	Position
NYT1	SMAD	E2F	CDE	CHR	Repressors
AAAGTT	GCTTAGAC	TGTCCCGC	GGGCGG	TTTGAAA	Sequence
-415/-410	-366/-358	-124/-116	-107/-102	-97/-91	Position

Figure 22. In silico promoter analysis of the rat Cks1b gene. (A) Cks1b gene location and annotation from the UCSC Genome Browser. Blue histogram depicts sequence conservation among vertebrates. Red bar signifies first coding region of Cks1b, green bar represents 5' untranslated region, dark blue bar represents the region of the Cks1b putative promoter under study, and light blue bar represents the first exon of the Shc1 gene. TSS = transcription start site. (B) Magnified genomic region -80/-135 (dark blue bar) shown in (A). TRANSFAC analysis of the region immediately upstream of the putative Cks1b transcription start site (TSS) revealed several potential transcription factor consensus sites. We focused on a pair of highly conserved sites, an E2F site (TGTCCCGC) and cell cycle-dependent element (CDE; GGCGGG) and cell cycle homology region (CHR; TTTCAAA) elements. E2Fs are family of transcription factors involved in a variety of functions including proliferation, DNA replication, and apoptosis. Both the CDE and CHR sites are involved in repression of genes whose expression is cell cycle-dependent. (C) A select list of transcriptiona factor consensus sites identified through TRANSFAC analysis listed by presumed function and position within the Cks1b promoter region relative to the TSS.

### E2F transcription factors are present in neural progenitor cells In vitro

To verify the presence or absence of E2F family members in NPCs, we performed reverse transcription PCR (RT-PCR) using cDNA made from neural progenitor cells isolated from E14 rat embryonic cortex (Figure 23). These cells were cultured in the presence of FGF2, and were passaged once (P1) after harvest. Parallel cultures of P1 neural progenitor cells were synchronized by withdrawal of FGF2 for 48 hours followed by re-application of FGF2 for 18 hours. For reverse transcriptase-PCR (RT-PCR), lysates were taken from FGF2-starved cultures (-FGF) and from cultures that were stimulated with FGF2 for 18 hours (+FGF2). As a positive control, cDNA was made from E14 rat whole embryos. We designed primers specific for E2F family members 1-6 and determined their relative amounts of mRNA by RT-PCR. As an additional positive control, we designed primers specific for the housekeeping gene cyclophilin A (Figure 23). Our RT-PCR data indicated that E2F1-6 mRNA is present in both proliferating and non-proliferating cultures, in the presence and absence of FGF2, respectively. This suggested that E2Fs might be functionally redundant in regulating Cks1b expression during the cell cycle of NPCs (Xu et al. 2007). Having identified which E2Fs are present in neural progenitors, we next wanted to demonstrate that E2Fs occupy the promoter of Cks1b in response to mitogen stimulation.



RT-PCR

**Figure 23.** E2F transcription factors 1-6 are present in both proliferating and nonproliferating NPCs. cDNA was made from E14 rat whole embryo or from NPC cultures in the presence or absence of FGF2. Primers specific for E2F1-6 were used in PCR for detection of transcript levels. Primers specific for cyclophilin A served as a positive control. No transcripts were detected in –RT samples.

### E2Fs occupy the Cks1b gene promoter at different phases of the cell cycle

Our in silico analysis data for the Cks1b gene promoter indicates that E2F transcription factors might be involved in regulating the expression of the Cks1b gene during cell cycle. To test directly this hypothesis, we used chromatin immunoprecipitation (ChIP) to enrich for the Cks1b gene promoter by E2F transcription factors in living cells. The strength of the ChIP assay is that it is the best method currently available to "visualize" directly an in vivo interaction between a specific DNA-binding protein and cis-regulatory element(s) for an endogenous gene.

In our assays, we used rabbit polyclonal antibodies that recognize mammalian E2F1 and E2F4 transcription factors to immuno-enrich for the Cks1b gene promoter. Several lines of evidence including the co-crystal structure and modeling of the E2F/DP/DNA complex predicts that all members of the E2F family are capable of binding the canonical E2F consensus motif in a similar manner. Thus, it is difficult to predict which family member (or members) bind to a specific E2F consensus in its native genomic context.

In these studies, we focused on E2F1 and E2F4, because of their common use by several investigators as representatives of activating and repressing E2Fs, respectively (Ren et al., 2002; Xu et al. 2007). In these series of experiments we include both negative and positive controls. For negative controls, we used: a) primers specific for the

βActin promoter, which does not contains any known E2F sites; b) a non-specific rabbit IgG to mock immuno-enrich for the Cks1b gene promoter to control for spurious enrichment due to unspecific binding; and c) PCR primers from sequence that lies 2000 bp upstream of the Cks1b TSS to control for spurious enrichment due to unsheared chromatin. For positive controls, we included the input sample, which is 2% of the total sheared chromatin used in each ChIP prior to immuno-enrichment, and we also enriched for the Cdc6 promoter, which is occupied by E2F4 and E2F1 in G0/G1 and S phase cells, respectively.

Although our focus is on the transcriptional regulation of the Cks1b gene in neural progenitor cells, we initially performed our ChIP experiments using HEK 293 and Rat2 fibroblast cell lines. Our reasons for doing so are threefold: a) it is likely that these regulatory mechanisms are common to other proliferating cell types besides NPCs; b) we can obtain more cells from a cell line than neural progenitors; and c) their progression through the cell cycle can be synchronized better than primary progenitors cells. Cells in culture progress through the cell cycle at different rates, so that they are asynchronous with respect to the phases of the cell cycle. Detection of cell cycle-related protein-DNA binding events is improved by enriching the culture for cells at the stage during which the particular event occurs. Serum starvation or low serum concentration prevents the progression of cells through the cell cycle, causing the majority of them to become quiescent in G0/G1, which ensures that upon re-application of serum, cells will begin passing synchronously through the cell cycle. Thus, synchronizing cell cultures is a technique for improving the signal to noise ratio in ChIP assays.

### The human Cks1b promoter is occupied by E2F4 and E2F1 during cell cycle in the HEK 293 fibroblast cell line

Synchronized HEK293 cells were collected at time points 0 (following 48 hour starvation period and just prior to serum stimulation), 3, 6, 9, and 18 hours after serumstimulation (Figure 24 A). DNA was cross-linked to protein using formaldehyde, and chromatin was then sheared to an average length of 500 bp through sonication to prevent immuno-enrichment of non-specific promoter regions (Figure 24 B). These time points were chosen because of their correspondence to specific phases of the cell cycle, with the onset of S phase at approximately 12 hours and onset of G2/M at approximately 24 hours following re-application of serum, according to FACS data (not shown). We showed by qualitative PCR that the repressor E2F4 enriches for the Cks1b gene promoter when cells are quiescent in G0/G1, and that E2F4 eventually dissociates from the Cks1b promoter approximately 3 hours post-stimulation (Figure 24 C). We did not detect E2F4 enrichment of Cks1b promoter at any other time-points tested. Conversely, E2F1 occupied the Cks1b promoter approximately 3 hours post-stimulation and remained bound throughout the cell cycle, and did not occupy the Cks1b promoter in quiescent G0/ G1 cells (Figure 24 C). We did not observe any Cks1b promoter enrichment in both the mock IP negative control and with primers specific for the  $\beta$ Actin promoter at any time point tested (Figure 24 C). The finding that both a repressor and activator E2F occupy the Cks1b promoter in G0/G1 and in cycling cells, respectively, is consistent with the predicted expression of Cks1b, initially low expression in G0/G1 then increasing levels to facilitate SCF<sup>Skp2</sup>-mediated destruction of p27<sup>KIP1</sup> promoting S phase transition. These findings are also consistent with the current model for E2F regulation of gene

transcription in which repressor E2Fs dominate occupancy of E2F-responsive target promoters in G0 and early G1, but are replaced by activator E2Fs that promote cell cycle progression upon entry and transition through the cell cycle (Attwooll et al., 2004).



**Figure 24.** The Cks1b promoter is bound by E2F4 and E2F1 at different phases of the cell cycle in HEK293 cells. (A) ChIP analysis was performed on synchronized HEK293 cells and lysates were taken at 0, 3, 6, 9, and 18 hours after serum-stimulation. (B) Cross-linked chromatin was sheared by sonication to an average fragment length of ~500 bp. Shown are pre- (P) and post- (S) sheared genomic DNA from the time points indicated. (C) Polyclonal antibodies against E2F4 and E2F1 were used to immuno-enrich for bound genomic DNA, while immunoprecipitation with a non-specific rabbit IgG served as a negative control. Input represents 2% of the total amount of chromatin added to each ChIP reaction. Primers specific for the Cks1b promoter flanking the E2F potential binding site were used in qualitative PCR to identify promoter occupancy of Cks1b by

either E2F proteins as cells transitioned through the cell cycle. As an additional negative control, primers were designed specific for the promoter of the Actin gene, which is not bound by either E2F1 or E2F4 proteins, and used in standard PCR analysis.

# DNA Content Analysis of a Rat2 fibroblast cell line synchronized by serum starvation by fluorescence activated cell sorting (FACS)

Based on our initial ChIP results in the human cell line, we next performed ChIP in synchronized Rat2 cells. The advantage of using Rat2 fibroblast cells is that it allows us to study E2F occupancy of the Cks1b promoter in a Rat cell type, but unlike rat neural progenitors, they are more amenable to synchronization through serum starvation and can be grown in large quantities for ChIP. For our ChIP experiments in Rat2 cells, we performed DNA content analysis, which allows us to monitor their cell cycle progression following stimulation with serum and will aid in our interpretations of the ChIP data. We made lysates at time-points corresponding to time-points used for ChIP and used propidium iodide (PI), which stains all double-stranded regions of both DNA and RNA by intercalating between the stacked bases of the double helix, for quantitative measurement of DNA by flow cytometry analysis. Rat2 cells were deprived of fetal bovine serum (FBS) for a period of 48 hours, at which point cultures were stimulated to re-enter the cell cycle by adding 20% FBS. We observed that, the majority of cells (~90%) were quiescent in G0/G1 after 48 hours without FBS. Most cells gradually shifted from G0/G1 to S phase, peaking (~40%) at 18 hours post-stimulation, ending up in G2/M phase around 24 hours post-stimulation (Figure 25 A).

#### Only E2F4 occupies the Cks1b promoter in synchronized Rat2 cells

For ChIP, we focused on two specific time points based on their correspondence to the cell cycle in Rat2 cells. We harvested cells at time point 0 that were quiescent in G0/G1, and also at 18 hours post-stimulation with serum, which is the point in the cycle when the largest percentage of cells are in S phase. We showed by qualitative PCR that, like in HEK293 cells, E2F4 was bound to the Cks1b promoter in quiescent cells in G0/ G1. However, unlike HEK293 cells, we did not detect any binding of E2F1 to the Cks1b promoter at any of the time-points tested (Figure 25 B). As a control, we performed qualitative PCR using primers specific to a region approximately 2 kb upstream from the E2F site in the Cks1b promoter, which was not enriched for by immunoprecipitation with either E2F4 or E2F1 antibody, suggesting that the enrichment we observe for the Cks1b promoter is real. We also enriched for the promoter region of Cdc6 that interacts with both E2F4 and E2F1 in G0 and S phase, respectively (Takahashi et al. 2000). The absence of enrichment of this PCR product suggests that the binding of E2Fs is specific to the proximal promoter region of Cks1b in these assays. We also analyzed the Rat2 ChIP data using real-time PCR to quantify more accurately the differences in E2F occupancy of the Cks1b promoter in the presence and absence of serum. The results from the real-time analysis showed a 17.5-fold change in promoter occupancy between the serum-deprived and serum-stimulated conditions, with greater enrichment of E2F4 for the Cks1b promoter in G0/G1, consistent with our findings from qualitative PCR (Figure 25 C).



**Figure 25**. The Cks1b promoter is bound by E2F4 in quiescent Rat2 cells. (A) Cell cycle distribution of Rat2 cells that were synchronized by serum deprivation. Cells were lysed at various time-points over a 24 hours period following serum stimulation and labeled with propidium iodide (pI) for DNA content analysis determined by flow cytometry. The y-axis represents the cell numbers and the x-axis represents the pI fluorescence intensity that translates to the quantity of DNA per cell. The leftmost peak represents G0/G1 cells while the rightmost peak represents cells in G2 and M phases, and the cells in between those peaks are in the S phase undergoing active DNA synthesis. (B) ChIP analysis was performed on synchronized Rat-2 cells and lysates were taken at 0 and 18 hours after serum-stimulation, which correspond to cells in G0/G1 and S phases of the cell cycle. Polyclonal antibodies against E2F4 and E2F1 were used to immuno-enrich for bound genomic DNA, while immunoprecipitation with a non-specific rabbit IgG served as a negative control. Input represents 2% of the total amount of chromatin added to each Primers specific for the Cks1b promoter flanking the E2F potential ChIP reaction. binding site were used in qualitative PCR to identify promoter occupancy of Cks1b by either E2F proteins as cells transitioned through the cell cycle. End-point PCR analysis revealed that E2F4 enriched for the Cks1b promoter exclusively in G0/G1 (F=4.793;  $p < 10^{-10}$ (0.05). Enrichment with the E2F1 antibody produce undetectable enrichment (F=0.04891; p > 0.05). As a negative control, primers specific for a sequence 2 kb upstream of the E2F site in the Cks1b promoter were designed and used in PCR analysis. No enrichment was detected with either E2F antibody. Both E2F4 and E2F1 are known to occupy the promoter of cdc6 gene in rodent cells, therefore we designed primers for this promoter, which served as a positive control for enrichment. (C) Rat2 ChIP samples were also analyzed by quantitative real-time PCR. Enrichment values for E2F4 and E2F1 for the Cks1b promoter were calculated as a percentage of total input, represented on the y-axis. The x-xis displays both the antibodies used in ChIP experiments and distinguishes samples that were either deprived (-) or stimulated (+) with FBS. Error bars represent standard error of the mean. Real-time PCR data are from three independent experiments. \* = p < 0.05.

Although the E2F4 binding of Cks1b in Rat2 cells was consistent with what we had observed in HEK293 cells, we did see a difference in E2F1 binding. This difference could be attributed to differences between species, which has been reported for certain E2F4 target genes (Conboy et al., 2007). Another explanation is that the binding of E2F1 to Cks1b may be tissue and cell type specific. (Odom et al., 2007).

## DNA Content Analysis by FACS of neural progenitor cells synchronized by FGF2 deprivation

While Rat2 cells allow us to monitor E2F occupancy of the endogenous rat Cks1b promoter, our goal is to investigate Cks1b promoter occupancy in NPC primary cultures in the presence or absence of FGF2. Synchronization and FACS analysis data from rodent NPCs in vitro are lacking. Therefore, to gain a better understanding of how the NPCs progress through the cell cycle in the absence or presence of FGF2 we performed DNA content analysis. Also this analysis will aid our interpretations of the ChIP data. Serum starvation has been widely used as a method for synchronizing cell lines. Primary cultures of neural progenitors, however, are maintained in serum-free media supplemented with FGF2. Therefore, we synchronized neural progenitor cells by depriving them of FGF2 for 48 hours, which forced the majority of them (83%) into a quiescent state in GO/G1. We did notice, however, that although the majority of cells (83%) were stopped in G0/G1 after 48 hours without FGF2, some cells continued to proliferate asynchronously, as indicated by the presence of a small G2/M peak. Still, most cells gradually shifted from G0/G1 to S phase, peaking (29%) at 18 hours poststimulation, ending up in G2/M phase around 24 hours post-stimulation (Figure 26 A).

## The Cks1b promoter is occupied by both E2F1 and E2F4 during cell cycle in rapidly dividing neural progenitor cells in vitro

In the ChIP experiments with neural progenitors, we focused on two time points, without FGF2 and 18 hours following FGF2 stimulation, that correspond to cells in G0/G1 and S phase, respectively. Because it is necessary to culture the NPCs at low density and ChIP requires large (2x10^7) quantities of cells, the NPC ChIP assays require a large

number of culture dishes and supporting material (for example, N2 media and FGF2 supplement) for each time point relative to the cell lines, therefore, we chose to focus on two points, at quiescence and at the peak of S phase in these cultures. Our data show that E2F4 enriched for the Cks1b promoter in quiescent cells in G0/G1, consistent with its role as a transcriptional repressor (Figure 26 B). Furthermore, the p130 pocket protein also enriched for the Cks1b promoter in G0/G1, which is consistent with the current model of E2F-mediated repression where the E2F4/p130 complex dominates the occupancy of target promoters in G0/G1 (Figure 26 B). The fact that we observed E2F4 occupancy of the Cks1b promoter across all cell types used in this study indicates that repression through E2F4 (and presumably p130) is likely a well-conserved mechanism for blocking Cks1b gene expression in non-dividing G0/G1 cells. We quantified these data using real-time PCR analysis that showed a 3.2-fold change and a 2.25-fold change in E2F4 and E2F1 promoter enrichment, respectively, between mitogen-starved and mitogen-stimulated NPCs (Figure 26 C).





Real-time PCR-ChIP



**Figure 26.** The Cks1b promoter is bound by E2F4 and E2F1 in response to mitogen signaling in NPCs in vitro. (A) Cell cycle distribution of NPCs that were cultured in the absence of FGF2 mitogen for 48 hours then stimulated to re-enter the cell cycle by addition of FGF2. Cells were lysed at various time-points over a 24 hours period following FGF2 stimulation and labeled with propidium iodide (pI) for DNA content analysis determined by flow cytometry. The y-axis represents the cell numbers and the xaxis represents the pI fluorescence intensity that translates to the quantity of DNA per cell. The leftmost peak represents GO/GI cells while the rightmost peak represents cells in G2 and M phases, and the cells in between those peaks are in the S phase undergoing active DNA synthesis. (B) ChIP analysis was performed on synchronized NPCs and lysates were taken at two time points, at quiescence (-FGF) and at 18 hours after FGF2 re-application. Polyclonal antibodies against E2F4 and E2F1, as well as p130 were used to immuno-enrich for bound genomic DNA, while immunoprecipitation with a nonspecific rabbit IgG serving as a negative control. Input represents 2% of the total amount of chromatin added to each ChIP reaction. Primers specific for the Cks1b promoter flanking the E2F potential binding site were used in qualitative PCR to identify promoter occupancy of Cks1b by E2Fs and pocket protein as cells transitioned through the cell cycle. (C) NPC ChIP samples were also analyzed by quantitative real-time PCR. Enrichment values for E2F4 and E2F1 for the Cks1b promoter were calculated as a percentage of total input, represented on the y-axis. The x-axis displays both the antibodies used in ChIP experiments and distinguishes samples that were either deprived (-) or stimulated (+) with FGF2. Error bars represent standard error of the mean. Realtime PCR data are from three independent experiments. No statistical significance enrichment for Cks1b was detected between cycling and non-cycling NPCS for E2F4 (F=2.745; p>0.05) or E2F1 (F=0.5389; p>0.05).

Using both qualitative and quantitative PCR, we observed occupancy of E2F4 to the Cks1b promoter quiescent NPCs, which is consistent with our findings in HEK293 and Rat2 cells. However, we also observed E2F4 occupancy in proliferating NPCs (Figure 26 B,C). This was also true of p130 enrichment (Figure 26 B). One possible interpretation of this result is that there may be cells that are unable to exit G0/G1 and reenter the cell cycle following stimulation with FGF2. If this were so, we would detect E2F4 occupancy from those cells at 18 hours post-stimulation. Another possible explanation is that, unlike serum synchronization in cell lines, NPCs aren't completely synchronized by withdrawing FGF2, resulting in asynchronously cycling cells that might contribute to spurious E2F4 and p130 enrichment of the Cks1b promoter (Figure 26 A). We also observed the presence of E2F1 binding in proliferating NPCs, whereas we were unable to detect binding in Rat2 cells. One possible explanation for this difference is that E2F1 occupancy of the Cks1b promoter may be cell-type specific; however, it is important to note that promoter occupation is not always indicative of regulatory function and may or may not translate into a functional relevant interaction.

#### In vitro transcriptional analysis of the rat Cks1b gene promoter

We used ChIP experiments to determine whether E2F proteins interact with the endogenous Cks1b gene promoter during the cell cycle. However, the ChIP assay cannot tell us whether this occupation is functionally relevant. Therefore, we performed a series of in vitro transcription activation assays that allowed us to directly test the functional significance of the E2F site. In addition we also targeted the CDE/CHR sites for Cks1b gene expression in response to FGF2. Like the E2F consensus site, both of these sequences are well conserved, and their positional proximity and orientation to the E2F consensus site suggests the existence of a potentially modular functional relationship (Odom et al., 2007). We therefore decided to include the CDE-CHR element in our mutagenesis experiments and measure its effect on the transcriptional activity of the Cks1b promoter.

#### Identification of the boundaries of the putative Cks1b gene promoter

Our strategy pertaining to the initial analysis of the rat Cks1b gene promoter was to identify the DNA elements necessary for Cks1b promoter activity following stimulation with serum and FGF2. We would predict that if serum and FGF2 do regulate the transcription of the Cks1b gene that re-application of these mitogenic factors to synchronized cells would cause an up-regulation in expression of the gene upon entering S phase, at approximately 12 hours after stimulation. If serum and FGF2 are not responsible for Cks1b transcription, we would predict that promoter reporter activity would remain at baseline (levels at quiescence) throughout the cell cycle following stimulation. We used PCR to isolate three fragments of the putative promoter region and fused them with a firefly luciferase reporter gene for subsequent luciferase assays (Figure 27 A). The longest fragment was ~1000 bp upstream of the transcription start site (TSS), spanning -1080 to -80 base pairs (bp) relative to the translation initiation codon. We chose this distance based on existing evidence that E2F motifs within target gene promoters show a positional bias relative to the transcription start site (TSS), and that evolutionarily conserved, functional E2F consensus sites are typically distributed within approximately 1000 bases of the TSS, often located within 100 bp and on either side of the TSS (Kel et al. 2001; Xie et al. 2005; Zhu et al. 2005). The transcription start site for the Cks1b gene had been mapped to a location approximately 80 bp upstream of the initiation codon according to the NCBI database, which is corroborated through the alignment of known expressed sequence tags (ESTs) to the 5' end of the Cks1b coding

sequence using the UCSC Genome Browser EST alignment tool. We also isolated an  $\sim$ 338 bp upstream of the TSS, spanning from -418 to -80 bp relative to the translation initiation codon. This fragment spans the genetic distance between the TSS of the Cks1b gene and TSS of the Shc1 gene, which is located ~338 bp upstream of the Cks1b TSS and transcribed in the opposite direction. We chose this fragment based on our earlier in silico promoter analysis that identified numerous well conserved putative transcription factor binding motifs within this segment (including the E2F and CDE/CHR consensus sites), as well as phylogenetic analysis of this region shows that it is highly conserved among mammals and among vertebrates whose genomes have been sequenced, all of which suggests that this relatively short segment might contain important regulatory elements controlling Cks1b gene expression. Finally, we isolated a ~478 bp fragment, spanning approximately -418 to +60 bp relative to the translation start codon. This fragment incorporates the genetic distance separating the Cks1b and Shc1 genes, as well as the 5' untranslated leader of the Cks1b gene, which often contains key regulatory elements, and the first 20 amino acids of the Cks1b open reading frame, which was included because of the evolutionarily conserved positional bias of E2F motifs mentioned above. These three promoter fragment luciferase reporter fusion constructs were expressed in synchronized HEK 293 cells through transient transfection and assayed for induction of luciferase activity in response to serum.



Figure 27. Promoter analysis of the rat Cks1b gene. (A) Schematic representing the correspondence of the promoter reporter constructs used in our luciferase assays with the We cloned a 1 kb (-1080/-80), a 338 bp (-418/-80), and a 478 bp rat Cks1b gene. (-418/+60) segment of the putative Cks1b promoter region and fused it to a firefly luciferase reporter. HEK293 cells were then co-transfected with one of the three Cks1b promoter reporters alongside a constitutively active renilla luciferase reporter driven by the CMV promoter. Cells were then synchronized by serum-starvation and lysates were collected at quiescence (0), 3, 6, 12, 18, 24, and 36 hours following serum-stimulation to measure luciferase activity. (B) Transcriptional activity for each reporter is represented as firefly luciferase activity normalized to renilla luciferase activity normalized to time point zero (y-axis). Normalized luciferase data for the firefly luciferase reporter under a constitutively active CMV promoter (CMV\_ffluc2) is also shown (maroon line). Each transfection was performed in triplicate. Error bars represent standard error of the mean.

## The 338 bp promoter fragment confers cell cycle-dependent Cks1b promoter activity

Although our primary objective was to identify the regulatory elements in the Cks1b gene promoter necessary for expression in neural progenitors in response to FGF2, we performed our initial promoter analysis in synchronized HEK 293 fibroblast cells because we think these regulatory mechanisms are conserved and because HEK 293 cells are easily transfected with DNA. Primary neural precursor cells are hard to transfect and we will need to utilize lentiviruses to perform these experiments (see bellow). We cotransfected HEK 293 cells with each of the three promoter-reporter constructs together with a constitutively active renilla luciferase reporter under a cytomegalovirus (CMV) promoter, which controls for variation in transfection efficiency and any other deviations due to cell culture conditions. We also controlled for induction artifacts by transfecting parallel cultures with a constitutively active firefly luciferase reporter under a CMV promoter (CMVffluc2). The CMV-driven firefly luciferase should show a minimal, if any, induction upon addition of serum/mitogen when normalized to the values from the CMV-driven renilla luciferase reporter. As a negative control, parallel cultures of HEK 293 cells were transfected with a promoterless firefly luciferase reporter. Each transfection was performed independently, and in triplicate. Transfected HEK 293 cells were synchronized by serum starvation followed by serum re-application, and lysates were collected at 0, 3, 6, 12, 18, 24, and 36 hours following serum stimulation. These specific time points were chosen because they correspond to different phases of the cell cycle as described above. In addition to our own cell cycle analysis, scientific literature in

this subject shows that time point 0 is when cells are quiescent in G0, and as cells are stimulated to progress synchronously through the cell cycle, time points 3 and 6 hours represent cells in G1, by 12 and 18 hours cells should have initiated and entered S phase, and at 24 hours cells should be progressing into G2/M phase. Also these are the same time points used in our ChIP assays, which allows us to make a direct comparison between E2F1 and E2F4 binding with promoter-reporter activity.

Data from these experiments indicated that all three promoter fragments were capable of driving the expression of the firefly luciferase gene after cell cycle re-entry in a manner that is consistent with what we would predict for the endogenous Cks1b promoter (Figure 27 B). The prediction would be that the level of Cks1b promoter activity is low initially in quiescent cells in G0/G1 and in early G1 coinciding with low levels of Cks1b gene expression and relatively high levels of p27<sup>Kip1</sup> activity. However, promoter activity should increase prior to the G1/S phase transition, when Cks1b expression is necessary for the destruction of p27<sup>Kip1</sup> through the SCF<sup>Skp2</sup> complex and successful initiation of S phase. In synchronized HEK 293 cells, levels of Cks1b promoter activity are low initially in quiescent G0/G1 and during early G1 phase up to 6 hours following re-application of serum at which point the promoter activity is rapidly induced between 6 and 12 hours, coinciding with the G1/S phase transition. This result is in sharp contrast to the activity of the firefly luciferase reporter under the constitutively active CMV promoter, which showed a relatively constant activation level throughout the cell cycle, as well as the promoterless firefly luciferase reporter, which was totally inactive at all time points measured. Although we observed slight differences in fold induction values among the promoter segments (approximately 5-fold, 6-fold, and 3-fold for the -1000/-80, -418/-80, and -418/+60 segments, respectively), all three promoter segments tested in this assay displayed a similar overall transcriptional profile (Figure 27 B), therefore we selected the minimal segment, the 338 bp promoter fragment spanning the intergenic region between Cks1b and Shc1, that contains the majority of conserved consensus elements including the E2F site, and used it for our mutagenesis studies.

We noticed that the -418/+60 promoter segment, which contains the intergenic sequence between Cks1b and Shc1 start sites as well as the 5'UTR and first coding exon of Cks1b, displayed the expected activation trend but was dampened compared to the -418/-80 segment. We reasoned that this difference in activation levels may be due to repressor cis-regulatory elements residing in the 5'UTR of the Cks1b gene. In silico analysis shows that a number of such elements exist within the 5'UTR as well as within the first coding exon. One such element, the NRSE repressor element, is located just downstream of the Cks1b TSS. NRSEs are bound by REST repressor transcription factors and are found in genes important for neurogenesis (Schoenherr et al., 1996; Ballas et al., 2005), however, we did not directly test (through mutagenesis) whether this element regulates the activity of the Cks1b.

# **Rationale** for mutating the E2F consensus and the cell cycle-dependent element (CDE) and cell cycle homology region (CHR) of the Cks1b promoter

We sought to directly test the roles of the E2F and CDE/CHR sites in regulating the expression of the Cks1b gene in response to serum/FGF2. To do this, we compared the induction profile of the wild type Cks1b gene promoter (spanning approximately -418 to -80 bp relative to the translation initiation codon) and mutant fusion constructs in luciferase reporter assays. For mutagenesis of the E2F site, we used overlap PCR to insert a 3 bp substitution mutation into the E2F consensus site (TGT<u>CCC</u>GCC  $\rightarrow$ TGTAGAGCC), which was based on a previously reported mutation of an identical E2F site within the upstream promoter region of the cyclin E gene (Le Cam et al., 1999). This muation is also consistent with what is known about the crystal structure of the E2F4/ DP2/DNA complex. The DNA-binding domains of the E2F and DP proteins recognize the central CGCGCG sequence within the target sequence TTTCGCGCG, and changing the core nucleotides c/gGC to c/gCC (TTT<u>CGCGCG</u>  $\rightarrow$  TTT<u>CCC</u>GCG) yielded similar affinity values (Slansky and Farnham, 1996; Zheng et al., 1999). This tri-nucleotide core, c/gGC or c/GCC is associated with an E2F4 amino-terminal extension (residues 16-19) that is conserved in the E2F family but not in the DP family, making it a potential target for both E2F4 and E2F1, as well as other members of the E2F family capable of forming heterodimers with DP proteins (Zheng et al., 1999). Based on these reported findings, as well as our own ChIP data, we predict that mutation of the E2F site will result in a perturbation of Cks1b promoter activity in the form of de-repression in quiescent cells in G0/G1 and attenuated activation upon entry into S phase.

A visual scan of sequences immediately upstream of the Cks1b TSS revealed the existence of consensus sites for the cell cycle-dependent element (CDE) and the cell cycle homology region (CHR), downstream of and in close proximity (within 50 bp) to the E2F consensus site within the Cks1b gene promoter. CDE/CHR-mediated repression is a common regulatory mechanism in cell cycle genes, and in most cases the CHR (5'-A/

TG/TGAA-3') cooperates with a contiguous CDE GC-rich motif (5'-G/CGCGG-3') to form a bipartite cell cycle-regulated repressor element (Zwicker et al., 1995). Inactivation of either component of this tandem module results in elevated transcriptional activity of CDE/CHR-regulated promoters in G0/G1 and throughout the cell cycle (Zwicker et al., 1995; Lucibello et al., 1995; Liu et al., 1996; Uchiumi et al., 1997). Like the E2F consensus site, both the CDE and CHR consensus sites in the Cks1b gene promoter are well conserved, and their positional proximity and orientation to the E2F consensus site suggests the existence of a potentially modular functional relationship (Elkon et al. 2003; Makeev et al. 2003; Odom et al., 2007). Consistent with this idea, it has been shown that E2F-mediated repression of the b-myb promoter is dependent on a contiguous co-repressor element that resembles a CHR consensus site (Bennett et al., 1996; Liu et al., 1996). We therefore decided to include the CDE/CHR element in our mutagenesis experiments and measure its effect on the transcriptional activity of the Cks1b promoter. We used overlap PCR to insert substitution mutations into the CDE/ CHR element (GGGCGG  $\rightarrow$  GATTAG and TTTGAA  $\rightarrow$  GCATAT, respectively) (Rother et al. 2007). We predict that mutation of the CDE/CHR element will result in increased activity of the Cks1b promoter reporter in quiescent G0/G1 cells that persists throughout the cell cycle. Furthermore, mutation of both the E2F and CDE/CHR consensus sites could potentially create a synergistic effect on Cks1b promoter activity in the form of enhanced de-repression in quiescent G0/G1 cells and throughout the cell cycle.

### E2F and CDE-CHR consensus sites in the Cks1b promoter are primarily repressor elements in HEK 293 cells

We hypothesized that the E2F and CDE-CHR consensus motifs contribute to the regulatory program(s) controlling the expression of the Cks1b gene. To directly test this hypothesis, we used luciferase promoter-reporter assays to compare the activity of the wild type Cks1b gene promoter (spanning approximately -418 to -80 bp relative to the initiation codon) versus the activity of the same promoter containing substitution mutations at either the E2F site (E2Fx) or the CDE-CHR sites (CDE-CHRx), in synchronized 293 and Rat2 fibroblast cell lines and in primary neural progenitor cultures Some evidence suggests that the co-existence of E2F and CDE-CHR sites in vitro. indicates a functional relationship between the two sites, which are in close proximity to one another in a number of cell cycle gene promoters, including the Cks1b promoter. Therefore, we included a third mutant construct, an E2F/CDE-CHR double mutation (Dblx), to test whether mutation of both elements has an additive de-repressive effect on promoter activity. Initial experiments were carried out in HEK 293 cells because these regulatory mechanisms are likely conserved from rat to human and because of the relative difficulty with transfecting Rat2 and neural progenitor cells. Exponentially growing HEK 293 cells were transiently transfected with promoter-reporter constructs and subsequently synchronized by serum starvation. We examined promoter activity at 3, 6, 9, 12, 18, 24, and 36 hours after serum re-application, which correspond to different phases of the cell cycle, as well as at time point 0 when cells are quiescent in G0/G1 phase. Based on the known functions of E2F family members, as well as our own ChIP
data, we would predict that mutation of the E2F consensus site would increase the transcriptional activity of the Cks1b promoter during G0/G1 and early G1, and decrease induction during the transition from G1 to S phase, coinciding with the disruption in binding of E2F repressors and activators, respectively. Because CDE-CHR motifs are predominately repressor elements in the promoters of cell cycle genes, we predict that mutating these sites will result in elevated transcriptional activation in quiescent G0/G1 cells. Finally, we predict that mutating both sites, the E2F and CDE-CHR, will have an additive effect, enhancing the de-repression of Cks1b promoter activity in quiescent cells in G0/G1.

Our data from 293 cells indicates that mutation of the E2F consensus site in the Cks1b promoter results in an increase in promoter activity in quiescent cells in G0/G1, which leads to an elevated level of activity throughout the cell cycle (Figure 28 A). In contrast, the activity of the CMV firefly luciferase reporter did not fluctuate during the cell cycle, confirming that the induction of promoter activity observed in these experiments is specific to the Cks1b promoter and not a general induction (Figure 28 A). These results are as predicted for a role as a repressor element in G0/G1 and early G1, but differed from what we predicted for the onset of S phase around 6 hours, suggesting that the E2F site does not play a functional role in the up-regulation of the Cks1b promoter during cell cycle progression. In other words, in 293 HEK cells E2F1 appears to interact with the promoter of Cks1b but seems not to be functionally relevant. However, it is also possible that the E2F1 binding seen in our 293 ChIP data occurs at an alternative, non-canonical E2F site elsewhere in the Cks1b promoter. Another possibility is that E2F1

may be 'poised' to occupy the Cks1b promoter, meaning that it is simply in close enough proximity to the Cks1b promoter that it is capable of enrichment, but does not make direct, functional contact. Similarly elevated levels of promoter activity were observed for the mutated CDE-CHR element (Figure 28 B). This finding is consistent with our prediction and with the known functional role of CDE-CHR consensus sites in cell cycle genes. Finally, the promoter activity of the double mutant construct was not significantly different from the activity of either mutant promoter alone (Figure 28 C), indicating that, in HEK 293 cells, the E2F and CDE-CHR sites do not cooperate to repress Cks1b promoter activity in the G0/G1 and the early phases of the cell cycle.



Figure 28. Mutation of either the E2F or CDE-CHR sites in the Cks1b promoter leads to increased activity of a luciferase reporter gene in response to serum stimulation in HEK 293 cells. We used a lipid-based plasmid delivery strategy to express the wild-type or mutated Cks1b promoter reporters in HEK 293 fibroblast cells. To control for variance in transfection efficiencies, a constitutively active renilla luciferase reporter (hRluc) was cotransfected alongside each promoter reporter. Cells were synchronized by serumstarvation and lysates were taken at quiescence (0), 3, 6, 12, 18, 24, and 36 hours following serum-stimulation to measure luciferase activity and is represented on the xaxis. Luciferase measurements are shown for the wildtype (WT) promoter reporter relative to the E2F (E2Fx; A), CDE-CHR (CDE-CHRx; B), and E2F/CDE-CHR (Dblx; C) mutant promoter reporters. A firefly luciferase gene under a CMV promoter (CMV\_ffluc2) was used as a positive control and is shown in (A). Luciferase activity was determined by normalizing the luciferase activity of the promoter reporter by a coinfected renilla reporter that is driven by a constitutively active CMV renilla luciferase promoter, represented on the y-axis. Error bars represent standard error of the mean (SEM). These data are from three independent experiments. ns = p > 0.05; \* = p < 0.05; \*\* = p < 0.001; \*\*\* = p < 0.0001.

## E2F and CDE-CHR consensus sites in the Cks1b promoter function primarily as repressor elements in Rat2 cells

Although our luciferase data from 293 cells were informative, our ultimate goal was to test our hypothesis in primary cultures of rat neural progenitor cells. Unlike HEK 293 cells, Rat2 and neural progenitor cells are more difficult to transfect through simple lipid-based delivery methods. Therefore, we used a retroviral delivery method with a lentiviral vector, which, in our hands, has been a more efficient delivery method for these difficult to transfect cells. Before proceeding to progenitor cells, however, we wanted to test the efficacy of each virus on a cell line. For this, we took advantage of a Rat2 fibroblast cell line that allowed us to test the efficacy of the virus in a rat cell type that is amenable to synchronization by serum starvation. We examined promoter activity at 3, 6, 12, 24, and 36 hours after serum re-application, which correspond to different phases of the cell cycle, as well as at time point 0 when cells are quiescent in G0/G1 phase. Prior

to infection, the number of viral particles was determined and an multiplicity of infection (MOI = (no. of viral particles/ single cell)) was calculated that was kept constant throughout the experiments. Based on the known functions of E2F family members, as well as our own ChIP data, we would predict that mutation of the E2F consensus site would increase the transcriptional activity of the Cks1b promoter during G0/G1 and early G1, but that induction will be like that of the wild-type promoter, since our ChIP data indicates that Cks1b is not a target of E2F1 in Rat2 cells. Because CDE-CHR motifs are predominately repressor elements in the promoters of cell cycle genes, we predict that mutating these sites will result in elevated transcriptional activation in quiescent G0/G1 cells. Finally, we predict that mutating both sites, the E2F and CHE-CHR, will have an additive effect, enhancing the de-repression of Cks1b promoter activity in quiescent cells in G0/G1.

Similar to our luciferase data in 293 cells, the data from Rat2 cells indicates that mutation of the E2F consensus site in the Cks1b promoter results in an increase in promoter activity in quiescent cells in G0/G1, which leads to an elevated level of activity throughout the cell cycle (Figure 29 A). Again, the activity of the CMV firefly luciferase reporter did not fluctuate during the cell cycle, confirming that the induction of promoter activity observed in these experiments is specific to the Cks1b promoter and not a general induction (Figure 29 A). These results are as predicted, indicating the E2F consensus site in the Cks1b promoter functions primarily as a repressor element in G0/G1 and early G1 in Rat2 cells. Also consistent with our ChIP data, the mutant E2F promoter was induced prior to S phase in a trend similar to that of the wild-type promoter, suggesting that E2F1

does not regulate Cks1b gene expression through the E2F consensus site. Similarly elevated levels of promoter activity were observed for the mutated CDE-CHR element (Figure 29 B). This finding is consistent with our prediction and with the known functional role of CDE-CHR consensus sites in cell cycle genes. Finally, the promoter activity of the double mutant construct was not significantly different from the activity of either mutant promoter alone (Figure 29 C), indicating that, in Rat2 cells, like that of HEK 293 cells (Figure 28 C), the E2F and CDE-CHR sites do not cooperate to repress Cks1b promoter activity in the G0/G1 and the early phases of the cell cycle. Unlike primary neural precursor cells, both HEK 293 and Rat2 cell lines are immortalized, or transformed, so that they have inherently altered growth properties allowing them to continue to grow and divide indefinitely in vitro under suitable culture conditions. Therefore, it is difficult to make a direct comparison between these cells and primary NPCs, as well as relate findings from those experiments to what might actually be happening in vivo.



Figure 29. Mutation of either the E2F or CDE-CHR sites in the Cks1b promoter leads to increased activation of a luciferase reporter gene in response to serum stimulation in Rat2 cells. We used a retroviral-based delivery system to infect Rat2 cells with wildtype and mutant Cks1b promoter reporter constructs, which were then split and plated to make multiple lysates. To control for variance in infection efficiencies, a constitutively active renilla luciferase reporter (hRluc) was co-infected alongside each promoter reporter. Cells were then synchronized by serum deprivation followed by re-application of 20% FBS. Lysates were harvested at the indicated time points that correspond to the phases of the cell cycle. Luciferase measurements are shown for the wildtype (WT) promoter reporter relative to the E2F (E2Fx; A), CDE-CHR (CDE-CHRx; B), and E2F/CDE-CHR (Dblx; C) mutant promoter reporters. A firefly luciferase gene under a CMV promoter was used as a positive control and is shown in D (red line). Luciferase activity was determined by normalizing the luciferase activity of the promoter reporter by a coinfected renilla reporter that is driven by a constitutively active CMV promoter. These data are from three independent experiments. ns = p > 0.05; \* = p < 0.05; \*\* = p < 0.001; \*\*\* = p < 0.0001.

### E2F and CDE-CHR consensus sites in the Cks1b promoter cooperate to regulate the cell cycle expression of the Cks1b gene in rat neural progenitor cells

The FACS and ChIP data from neural progenitors indicated that E2F1 and E2F4 occupy the Cks1b promoter at different points in the cell cycle, either in the presence or absence of FGF2, respectively. To test whether the E2F consensus site in the Cks1b promoter is a target of activator and repressor E2F proteins in quiescent and cycling neural progenitors in vitro, we infected neural progenitor cultures with the same viral constructs described above for Rat2 cells and measured the luciferase activity throughout cell cycle progression. As discussed previously, unlike 293 and Rat2 cell lines, there is no established method for synchronizing neural progenitor cultures, or for synchronizing most primary cultures in general. To synchronize neural progenitor cultures, we removed FGF2 from the media for 48 hours, which DNA content analysis suggests drives NPCs into quiescence in G0/G1. We examined promoter activity at 3, 6, 12, 24, and 36 hours after re-application of FGF2, which correspond to different phases of the cell cycle,

as well as at time point 0 when cells are quiescent in G0/G1 phase. Prior to infection, the number of viral particles was determined and an multiplicity of infection (MOI = (no. of viral particles/ single cell)) was calculated that was kept constant throughout the experiments. Based on the known functions of E2F family members, as well as our own ChIP data that shows the Cks1b promoter is a target of both activator and repressor E2Fs, we would predict that mutation of the E2F consensus site would play a dual role, both in increasing the transcriptional activity of the Cks1b promoter during G0/G1 and in early G1, and disrupting induction of promoter activity during the transition into S phase. Because CDE-CHR motifs are predominately repressor elements in the promoters of cell cycle genes, we predict that mutating these sites will result in elevated transcriptional activation in quiescent G0/G1 cells. Finally, we predict that mutating both sites, the E2F and CDE-CHR, will have an additive effect, enhancing the de-repression of Cks1b promoter activity in quiescent cells in G0/G1, but that the effect on induction will be similar to the E2F mutant alone.

The data from neural progenitor cells indicates that mutation of the E2F consensus site in the Cks1b promoter results in an increase in promoter activity in quiescent cells in G0/G1, which led to an elevated level of activity throughout the cell cycle until approximately 24 hours after FGF2 stimulation, at which point the activity began to decrease (Figure 30 A). A similar transcriptional profile was observed for the mutated CDE-CHR reporter (Figure 29 B). The promoter activity of the double mutant, however, produced both an increased activation at early points within the cell cycle and a delayed onset of activation later in the cell cycle (Figure 30 C). We had predicted that

mutation of the E2F site would disrupt the occupation of this site by both activator and repressor E2F proteins, essentially rendering the Cks1b promoter unresponsive to cell cycle phase transitions. However, it was only in the presence of both the E2F and CDE-CHR mutations did we see this effect, suggesting that some cooperation exists between these two consensus elements in regulating Cks1b gene expression in neural progenitors. Interestingly, the induction is only delayed, not abolished, suggesting that other cisregulatory elements are involved in Cks1b gene expression (Figure 30 C). Finally, the activity of the CMV firefly luciferase reporter did not fluctuate during the cell cycle, confirming that the induction of promoter activity observed in these experiments is specific to the Cks1b promoter and not a general mechanism of induction (Figure 30 A). We did, however, note that the activity of the CMV promoter is much weaker than that of the Cks1b promoter, which was only observed in neural progenitor cells and not in either of the cell lines (see Figure 28 A and Figure 29 A).



Figure 30. Mutation of either the E2F or CDE-CHR sites in the Cks1b promoter leads to increased activation of a luciferase reporter gene in G0/G1 and delayed activation of S phase in response to mitogen stimulation in NPCs. We used a lipid-based plasmid delivery strategy to express the either wild-type or mutant Cks1b promoter reporters in non-adherent P1 neurospheres. The next day neurospheres were plated onto dishes coated with fibronectin and 3 hours later FGF2 was withdrawn from the media. NPCs were stimulated 48 hours later by adding back FGF2 to the media. Lysates were captured at each indicated time point corresponding to the different phases of the cell cycle and used in a dual luciferase assay according to the manufacturer's protocol. Luciferase measurements are shown for the wildtype (WT) promoter reporter relative to the E2F (E2Fx; A), CDE-CHR (CDE-CHRx; B), and E2F/CDE-CHR (Dblx; C) mutant promoter reporters. A firefly luciferase gene under a CMV promoter (CMV\_ffluc2) was used as a positive control and is shown in (A). Luciferase activity was determined by normalizing the luciferase activity of the promoter reporter by a co-infected renilla reporter that is driven by a constitutively active CMV promoter (hRluc). These data are from three independent experiments. ns = p > 0.05; \* = p < 0.05; \*\* = p < 0.001; \*\*\* = p < 0.0001.

### The E2F consensus site mediates E2F4 binding to the Cks1b promoter in G0/G1

To further consolidate a causal link between the E2F consensus in the Cks1b promoter and E2F4 binding we performed reporter ChIP assays. This experiment is done by using ChIP assays that measure protein-DNA interactions with transfected promoter sequences rather than with endogenous DNA promoter elements (Giangrande et al., 2004). Using this modified ChIP assay, we can also measure the effect of specific mutations on the E2F consensus by the E2F4 (Figure 31 A). Specifically, we used the Cks1b reporter plasmid for these assays and measured the interaction of E2F4 with the reporter in quiescent HEK 293 cells. We chose this specific time point based on the ChIP and luciferase data, which suggests that E2F4 occupies the E2F consensus site in the Cks1b promoter and represses Cks1b transcription in G0/G1 cells. Furthermore, the luciferase data in 293 cells suggests that the E2F site in the Cks1b promoter plays little or no role in activation of Cks1b gene in S phase. The promoter carrying a mutation at the

E2F consensus site used in this experiment is the same mutant promoter used in our luciferase assay. Samples of fibroblasts transfected with either the wild-type or E2F mutant Cks1b promoter constructs were assayed for interaction of E2F4 with reporter DNA. For PCR analysis, we designed primers that recognized sequences specific to the promoter construct, specifically, the reverse primer is specific for sequence within the luciferase open reading frame (Figure 31 B). This design allows us to measure only the transfected sequences and not enrichment of the endogenous promoter sequence. Using both end-point and real-time PCR analyses, we show that E2F4 binds to the wild-type promoter in G0/G1-arrested cells (0h) as was the case for the endogenous ChIP assays above. Assays of the mutant promoter revealed that disruption of the E2F consensus site interferes with E2F4 binding to the reporter DNA (3.6-fold change) (Figure 31 C). Based on these results, we conclude that the E2F consensus site is a critical node for E2F4 occupancy of the Cks1b promoter and therefore plays an important role in repression of Cks1b gene activation in G0/G1 phase.



**Figure 31**. Mutation of the E2F consensus site reduces the promoter occupancy of the Cks1b promoter by E2F4 in HEK293 cells in G0/G1. (A) HEK293 cells were transfected with 12ug of either the wildtype or E2F mutant promoter reporter plasmids alongside a renilla luciferase under a CMV promoter at a ratio of 7:1. 24 hours later cells were split 1:3, and 24 hours after splitting serum was removed from the media and cells were synchronized by serum starvation for 48 hours, at which point lysates were made and

processed for ChIP. (B) Schematic showing the placement of primers used in PCR analysis relative to the E2F site for both the wildtype and E2F mutant Cks1b gene promoters. (C) Reporter ChIP (rChIP) DNA was analyzed by both qualitative and real-time PCR. PCR data showed that only the wildtype Cks1b promoter reporter was enriched for by the E2F4 antibody in HEK293 cells in G0/G1. No E2F4 enrichment was observed in rChIP DNA from cells that were transected with exogenous promoter carrying the E2F mutation. In the graph, the y-axis represents the enrichment of E2F4 for either the Cks1b wild-type or E2F mutant promoter-construct as a proportion of total input. These values represent data from three independent experiments.

## p27<sup>Kip1</sup> expression is not sufficient to promote neuronal differentiation of neural progenitors in the VZ

It has been argued that specific cell cycle regulators have cell–cycle independent functions. For example several reports have shown that  $p27^{Kip1}$  promotes differentiation of neurons in frogs and mice. However, mutant mice that have deletions in the  $p27^{Kip1}$  or some of the components of the SCF complex like Skp2 and Cks1b do not display any overt phenotypes in the CNS. To explore whether  $p27^{Kip1}$  has an independent role in neuron differentiation we used a gain-of-function approach in chick embryonic spinal cords. We ectopically expressed chicken  $p27^{Kip1}$  by using in ovo electroporations at Hamburger-Hamilton (HH; Hamburger and Hamilton, 1951) stage 10-12 and analyzed phenotypic changes within the spinal cord 48-72 hours later.

We used immunohistochemistry of label sections from transfected HH24-25 embryos to determine whether ectopic expression of p27<sup>Kip1</sup> is sufficient to alter the processes of CNS development. To address this, the cell cycle inhibitor p27<sup>Kip1</sup> was ectopically expressed in the chick neural tube. Expression of p27<sup>Kip1</sup> within the VZ did not induce differentiation in GFP-positive cells within the ventricular zone as evidence by lack of expression of lineage-specific neuronal markers like NeuN (Figure 32 A-C), Tuj1 (Figure 32 D-F), and HuD (Figure 32 G,H). While the transfected halves of the spinal cords showed a dramatic reduction in overall size, ectopic expression of  $p27^{Kip1}$  did not alter the fates of neural cells during the period of neurogenesis. Furthermore, expression of Pax7 (Figure 33 A-C) and Pax6 (Figure 33 D-F), two members of the homeobox family of transcription factors that are specifically expressed in progenitor domains in the developing neural tube was unaltered in the transfected side of the spinal cord, relative to non-transfected side of the spinal cord. These findings that forced cell cycle exit through the ectopic expression of  $p27^{Kip1}$  does not influence the fate decisions of neural progenitors, nor does it alter their underlying transcriptional code, suggests that  $p27^{Kip1}$  alone is not sufficient for cellular differentiation of neural progenitor cells in vivo.



**Figure 32.** p27<sup>Kip1</sup> does not effect neuronal fate specification in the developing chick neural tube. An in ovo electroporation strategy was used to ectopically express p27<sup>Kip1</sup> in the developing neural tube of stage HH10-12 chick embryos. Following electroporation, embryos were allowed to develop and were sectioned at stage HH24-25. Electroporated embryos were probed with antibodies for the neuronal-specific markers NeuN (A-C), ßIII-tubulin (Tuj1) (D-F), and HuD (G,H). The white bar at the bottom right corner of micrograph H represents 100 μm.



**Figure 33.** Forced expression of p27<sup>Kip1</sup> does not alter the transcriptional code in the developing chick neural tube. An in ovo electroporation strategy was used to ectopically

express  $p27^{Kip1}$  in the developing neural tube of stage HH10-12 chick embryos. Following electroporation, embryos were allowed to develop and were sectioned at stage HH24-25. Antibodies specific for Pax 7 (D-F) and Pax6 (A-C) showed that progenitor domains in the developing neural tube were unaltered in transfected embryos. White bar in bottom right corner of (F) represents 100 µm.

Ectopic expression of p27<sup>Kip1</sup> did, however, have a significant effect on the number of proliferating cells in the ventricular zone. BrdU pulse labeling for 30 min of electroporated embryos revealed that overexpression of p27Kip1 reduced the total number of proliferating cells in the ventricular zone in the transfected side of the spinal cord (Figure 34 A-D). There was also a noticeable reduction in the number of VZ progenitors expressing phospho-histone 3 (pH3) (Figure 34 E,F) and MPM2 (Figure 34 G-I), two markers that label dividing cells in G2/M phase, in the transfected side versus the nontransfected side of the spinal cord. Since this reduction in overall size of the spinal cord and lack of proliferating progenitors could be attributable to increased neuronal cell death due to overexpression of the plasmid or the damaged caused by the electroporation technique itself, we probed for apoptosis using the TUNNEL method. We were unable to detect any noticeable difference in the extent of cell death in the transfected versus nontransfected halves in the spinal cords of electroporated embryos (Figure 35 A,B). As a positive control, we used deoxyribonuclease 1 (DNase 1), which is required for intranuclear cleavage of DNA during apoptosis (Figure 35 C). These findings, taken as a whole, suggest that during neurogenesis, p27Kip1 has a functional role in cell cycle withdrawal of neural progenitors but does not specify neuronal fates.



**Figure 34.** Forced expression of  $p27^{Kip1}$  reduced the number of proliferating cells in the developing chick neural tube. An in ovo electroporation strategy was used to ectopically express  $p27^{Kip1}$  in the developing neural tube of stage HH10-12 chick embryos. Following electroporation, embryos were allowed to develop and were sectioned at stage HH24-25. Prior to dissection, embryos were treated with a pulse of BrdU and incubated for 30 min. Embryos were then sectioned and stained for detection of BrdU. A decrease in the number of proliferating cells was observed within the VZ, indicated by decreased BrdU (A-C, quantified in D), and a decrease in phosphorylated Histone-H3 expression (E,F), and MPM-2 (G-I) expression in the transfected side versus the non-transfected side. White bar in bottom right of (I) represents 100 µm.



**Figure 35.** Forced expression of  $p27^{Kip1}$  does not cause an increase in programmed cell death in the developing chick neural tube. TUNEL staining was performed to assess the level of apoptosis after ectopic expression of  $p27^{Kip1}$  by in ovo electorporation. The amount of cell death in embryos over-expressing  $p27^{Kip1}$  was indistinguishable from mock transfected embryos (A,B), suggesting that the reduction in the overall size of transfected side of electroporated spinal cords is not attributable to increased cell death (D-F). DNase I served as a positive control in our TUNEL assays (C). White bars at bottom right of (C) and (F) represent 100 µm.

#### Chapter III

#### **GENERAL DISCUSSION**

## I. p27<sup>Kip1</sup> protein expression is associated with post-mitotic neurons but not ventricular zone progenitor cells during early neurogenesis

In this study, we investigated how neural precursor cells coordinate cell proliferation and cyto-differentiation through regulation of the cell cycle. We hypothesized that during embryogenesis, neural precursor cells in the ventricular zone maintain their proliferation in response to growth factors by repressing the cell cycle inhibitor p27<sup>Kip1</sup>, in part, through the up-regulation of Cks1b and activation of the degradation complex SCF<sup>Skp2</sup>. To determine what role p27<sup>Kip1</sup> might play in regulating cell cycle progression and withdrawal of neural progenitors during neurogenesis, we first characterized the expression pattern of p27Kip1 mRNA and protein during this developmental period in chicken, mouse, rat, and human CNS. We report that while p27<sup>Kip1</sup> mRNA is detected in the ventricular zone and in the mantle zone of the spinal cord and cortical plate of the cerebral cortex, p27Kip1 protein was mostly absent from the VZ throughout the neuraxis. This was true in chicken, mouse, rat, and human CNS tissues, and is consistent with what has been reported elsewhere for the expression pattern of p27<sup>Kip1</sup> in the developing nervous system (van lookeran et al. 1998; Nagahama et al. 2001; Nguyen et al. 2006; Gui et al. 2007). In the forebrain of E12 rat, Lee et al. (1996) described weak p27<sup>Kip1</sup> immunoreactivity in the ventricular zone of the cerebral cortex, a layer containing mostly proliferating neuroblasts, and relatively strong p27<sup>Kip1</sup> staining in the cortical plate and preplate which mostly contain post-mitotic neurons. The high level of p27Kip1 immunoreactivity was observed in essentially all cells in the cortical plate and also in neurons of the basal telencephalon and diencephalon, suggesting that high  $p27^{Kip1}$  levels are characteristic of post-mitotic neurons (Lee et al. 1996). Nguyen et al. (2006) also reported that a small fraction of cells in the VZ/SVZ of the cerebral cortex of embryonic mice were positive for  $p27^{Kip1}$ . We found that the relatively small number of  $p27^{Kip1}$ -positive cells in the VZ, in either the brain or spinal cord, express lineage-specific markers like IB4, an endothelial marker that identifies blood vessels,  $\beta$ III tubulin (Tuj1) or HuD, two neuronal lineage-specific markers. The co-expression of  $p27^{Kip1}$  and Tuj1 and HuD suggests that these cells are immature neurons that have not yet begun to, or are in the process of, migrating out of the VZ.

# **II.** A potential compensatory role of the Cip/Kip proteins in cell cycle exit in neural precursor cells during development

The ability of neural progenitor cells to exit the cell cycle in the absence of p27<sup>Kip1</sup> suggests that other cell cycle inhibitors are involved. The phenotypes of mice deficient in each of the three CKIs suggest that p21<sup>CIP1</sup> is not essential for development, whereas p27<sup>KIP1</sup> and p57<sup>KIP2</sup> play important roles in development, probably by limiting cell proliferation (Nakayama and Nakayama, 1998). In the adult, p21-deficient mice have more NSCs than their wildtype counterparts due to higher proliferation rates of neural stem cells, and while both p21-/- and wildtype NSCs retain the capacity for self-renewal in vitro, p21-/- NSCs display limited in vitro capacity for self-replication, suggesting that p21<sup>CIP1</sup> is necessary for the long term maintenance of NSC self-renewal (Kippin et al. 2005). p27-deficient mice are larger than their wildtype littermates, a result attributed to unregulated proliferation and tissue growth, but are otherwise normal

(Nakayama et al., 1996; Kiwokawa et al., 1996; Fero et al., 1996). Mice lacking p57KIP2 die immediately after birth due primarily to a severe cleft palate, abdominal muscle defects and skeletal abnormalities due to defects in endochondral ossification (Zhang et al. 1997; Yan et al. 1997; Takahashi et al. 2000). p27-/- p57-/- double mutants mice show defects in cell-cycle exit of neural progenitors that are associated with a reduction in the generation of ventral interneurons (Gui et al., 2007). The three members of the Cip/Kip family display distinct spatial and temporal expression patterns in the developing mouse spinal cord (Gui et al., 2007). All three Cip/Kip proteins are expressed in various neuronal subtypes during neurogenesis in the spinal cord, with both p21<sup>CIP1</sup> and p57<sup>KIP2</sup> being expressed briefly in the nuclei of distinct sub-classes of interneurons leaving the VZ, while p27<sup>KIP1</sup> expression is initiated and maintained in the nuclei of post-mitotic neurons in the mantle zone (Gui et al. 2007). In most tissues, the expression of  $p57^{KIP2}$ ceases or is reduced dramatically before birth, whereas p27<sup>KIP1</sup> expression perpetuates into adulthood, suggesting that p57<sup>KIP2</sup> is probably more important in early morphogenesis, whereas p27<sup>KIP1</sup> might be important in development and for maintenance some adult tissues (Nagahama et al. 2001).

Among the Cip/Kip family of CKIs, p27<sup>KIP1</sup> and p57<sup>KIP2</sup> show the highest degree of structural similarity, as both share a conserved CDK binding-inhibitory domain and a QT domain in their N- and carboxy-terminal regions, respectively, while p21<sup>CIP1</sup> lacks a QT domain (Lee et al. 1995; Matsuoka et al. 1995). The expression levels of both p27<sup>KIP1</sup> and p57<sup>KIP2</sup> are high in G0 and G1 phases of the cell cycle and decrease in association with the activation of cyclin-CDK complexes and progression from G1 to S

phase, and overexpression of either p27KIP1 or p57KIP2 in cultured cells induces G1 arrest (Susaki et al., 2009). Mice engineered to express increasing and decreasing levels of p27KIP1 indicate that the levels of this CKI critically regulate the fraction of precursors that re-enter S phase (Cunningham and Roussel, 2001; Caviness et al., 2003). In our in ovo experiments, ectopic expression of p27Kip1 dramatically depleted the number of proliferating cells in the spinal cord, leading to a diminution of undifferentiated cells in the precursor pool and ultimately a reduction in the overall size and shape of the transfected side of the spinal cord. Thus, while overexpression of  $p27^{Kip1}$  seems capable of forcing cells to exit the cell cycle, endogenous p27Kip1 alone appears to be dispensable for regulation of cell cycle progression in neural progenitors. However, it should be noted that while divisions of neural progenitors are confined to the innermost cell laver, the ventricular zone (VZ), and to the adjacent, telencephalon-specific, subventricular zone (SVZ) (Smart, 1973; Rakic, 1988; Gotz and Huttner, 2005; Kriegstein et al. 2006), no SVZ has been described, and no intermediate progenitors have been shown to reside in the developing spinal cord (however, in the adult mouse spinal cord, both an ependymal and subependymal layer has been described (Martens et al. 2002)). Although we did not test this directly, it is possible that more neurogenic intermediate progenitors (as opposed to the more proliferative apical progenitors), residing at the lateral edge of the ventricular zone, begin expressing p27<sup>KIP1</sup> as they prepare to exit the cell cycle and migrate away from the VZ.

# III. p27<sup>KIP1</sup> protein is either nuclear, cytoplasmic, or both depending on the developmental stage and axial level of the developing neural tube

Originally studied for its role in regulation of cell cycle, p27<sup>KIP1</sup> has emerged as a multifaceted protein with functions beyond cell cycle exit. The multifunctionality of p27<sup>KIP1</sup> relies on the existence of different domains in the molecule that independently regulate distinct pathways. The N-terminus of p27<sup>KIP1</sup> protein contains the cell cycle regulatory function, where p27<sup>KIP1</sup> inhibits the activity of cyclin E/A-CDK2 preventing the G1/S phase transition, while the carboxy-terminus of p27<sup>KIP1</sup> is necessary for promoting radial migration (Nguyen et al. 2006). The mechanism underlying the role of p27<sup>KIP1</sup> in migration appears to involve the activation of cofilin, an actin-binding protein that promotes actin reorganization and has been shown to be important for proper cortical neuronal migration (Moriyama et al., 1996; Gungabissoon and Bamburg, 2003). Cdk5 stabilizes p27<sup>KIP1</sup> through phosphorylation of the Ser10 phosphosite on p27<sup>KIP1</sup>. This increase in p27<sup>KIP1</sup> protein levels negatively regulates the phosphorylation of cofilin leading to its activation through suppression of the small GTPase RhoA by interfering with RhoA binding to its guanine-nucleotide exchange factors (GEFs) (Besson et al. 2004; Kawauchi et al. 2005).

We show that the subcellular localization of  $p27^{KIP1}$  in neurons depends on both the developmental stage of the embryo and axial level of the developing spinal cord. We find that at earlier developmental stages like E12 rat  $p27^{KIP1}$  appears almost entirely nuclear throughout the mantle zone, while at E14.5  $p27^{KIP1}$  is localized in the cytoplasm in the dorsal half of the spinal cord, more nuclear in ventrally located motor neurons, and in both the cytoplasm and nucleus at points in between (ventral midline, for example). Three phosphorylation sites on p27KIP1 mediate its localization in the cytoplasm. Phosphorylation of p27<sup>KIP1</sup> at the phosphosite Ser10 stabilizes p27<sup>KIP1</sup> protein in quiescent cells, leading to its nuclear export to the cytosol in G1 phase via CRM1/ exportin1 (Ishida et al., 2000; Rodier et al., 2001; Besson et al., 2006). A number of kinases are thought to be involved in p27<sup>KIP1</sup> phosphorylation at Ser10. In quiescent cells, the Mirk/dyrk1B kinase has been proposed to phosphorylate this site (Deng et al., 2004), whereas human stathmin (hKIS) has been linked to Ser10 phosphorylation at the G0/G1 transition in response to mitogen stimulation (Boehm et al., 2002). Other kinases like PKB/Akt, and extracellular signal-regulated kinase-2 (ERK2) have been shown to phosphorylate Ser10 in vitro (Ishida et al., 2000; Fujita et al., 2002). Phosphorylation of other phosphosites on p27<sup>KIP1</sup> like Thr-157 or Thr-198 by PKB/Akt or p90 ribosomal S6kinase (p90RSK) causes p27<sup>KIP1</sup> to be sequestered in the cytosol by promoting the association of p27KIP1 with 14-3-3 protein, which prevents nuclear translocation of p27<sup>KIP1</sup> by disrupting its interaction with importin alpha (Liang et al., 2002; Fujita et al. 2003; Sekimoto et al., 2004).

The finding that p27<sup>KIP1</sup> protein is present in both cytoplasm and nucleus, in different ratios at different stages of differentiation, suggests that it likely regulates different processes in different cellular compartments, interacting with cyclin/CDKs in the nucleus while interacting with RhoA in the cytoplasm. In the developing rat spinal cord, neurogenesis follows at least three gradients: rostro-caudal, ventral-dorsal, and lateral-medial (Bayer and Altman, 1982). Thus, the predominately cytoplasmic localization of p27<sup>KIP1</sup> in early embryos (early neurogenesis) and in more dorsal aspects

of the spinal cord in older embryos likely reflects the ventral-to-caudal gradient of neurogenesis, wherein  $p27^{KIP1}$  is more nuclear in more mature, post-mitotic neurons in order to aid in preventing cell cycle reentry, whereas  $p27^{KIP1}$  is mostly cytoplasmic in more newly-born neurons located more dorsally, perhaps indicative of a role in neuronal migration, as these immature neurons may or may not be completely post-mitotic, and may be in the process of migration out of the VZ. Interestingly, both Nguyen et al. (2006) and Gui et al. (2007) reported similar findings, that the subcellular localization of  $p27^{KIP1}$  is predominately nuclear in post-mitotic neurons in the mantle layer of the developing mouse spinal cord (Gui et al. 2007) and cortical plate of the cerebral cortex (Nguyen et al. 2006). In the mouse cerebral cortex in particular,  $p27^{KIP1}$  was reported to shift from predominately cytoplasmic subcellular localization in VZ and IZ progenitors to a predominately nuclear localization in CP neurons (Nguyen et al. 2006), suggesting a cell cycle-independent function of  $p27^{KIP1}$  in progenitors and migrating neuroblasts.

We report that  $p27^{KIP1}$  is either nuclear, cytoplasmic, or both depending on the developmental stage or level of the neuroaxis. For example,  $p27^{KIP1}$  is predominately nuclear in motor neurons in the lumbar region of the spinal cord, but this localization becomes much more inconsistent at more thoracic and cervical levels. This may reflect that in more mature neurons,  $p27^{KIP1}$  is more compartmentalized in the nucleus allowing it to block cell cycle progression (discussed below). In more dorsally located neurons,  $p27^{KIP1}$  may be compartmentalized more in the cytoplasm in order to facilitate its interaction with cytoskeletal regulatory proteins thereby promoting migration in younger, more motile neurons. This is contrast to earlier developmental expression of  $p27^{KIP1}$  in

the spinal cord and cerebral cortex, where p27<sup>KIP1</sup> is predominately nuclear throughout the ventricular neuraxis, where it likely plays a larger role regulating cell cycle progression.

We also detected predominately nuclear  $p27^{KIP1}$  in DRG sensory neurons at all levels of the neuraxis, and that  $p27^{KIP1}$  is expressed in axons within the ventral root, as well as in ascending funiculi and crossing fibers. The presence of  $p27^{KIP1}$  in axons has not been reported previously, and to our knowledge is a novel finding. Although we did not address this question directly in our study, it is tempting to speculate on the role of  $p27^{KIP1}$  in axons, particularly it's C-terminal domain, which has been shown to interact with RhoA and members of actin cytoskeletal regulatory proteins. RhoA is an important small GTPase that plays a particularly prevalent role in growth cone collapse in response to inhibitory guidance cues. It is known that all three Cip/Kip proteins are capable of interacting with cytoskeletal regulators, inhibiting the Rho/Rho-kinase (ROCK)/LIMkinase (LIMK)/Cofilin signaling pathway, albeit acting at distinct levels (Besson et al., 2004, 2008). Regulation of axonal growth and guidance in the developing spinal cord may be either a unique feature of  $p27^{KIP1}$  or perhaps a general mechanism through which all Cip/Kip's play a role.

## IV. Up-regulation of Cks1b in response to mitogenic factors maintains low levels of p27<sup>Kip1</sup> expression in proliferating cells

The absence of p27<sup>Kip1</sup> protein in proliferating cells within the ventricular zone despite the strong presence of p27<sup>Kip1</sup> mRNA suggests that neural progenitor cells regulate p27<sup>Kip1</sup> protein expression at the post-transcriptional level. Previous work has

shown that p27<sup>KIP1</sup> is regulated mostly at the post-translational level (Hengst and Reed, 1996), through the kinase activity of cyclin E/A-CDK2 that phosphorylates p27<sup>KIP1</sup> at residue Thr-187, initiating ubiquitin-mediated proteasomal degradation of p27KIP1 (Pagano et al., 1995; Sheaff et al., 1997; Carrano et al., 1999; Sutterluty et al., 1999; Montagnoli et al., 1999; Tsvetkov et al., 1999). The SCF<sup>Skp2</sup> E3 ligase is primarily responsible for the polyubiquitination of p27KIP1 and therefore plays a key role in regulation of cell cycle progression, facilitating proliferation through the destruction of p27<sup>KIP1</sup>. We have shown that levels of mRNA and protein for Cks1b and Skp2, the two components of the SCF<sup>Skp2</sup> complex that are responsible for specifically targeting p27<sup>KIP1</sup> for polyubiquitination, are highly expressed by neural progenitors throughout the ventricular neuraxis, and are both largely absent from neuronal populations in the mantle layer and cortical plate of the spinal cord and brain, respectively. In addition, we have shown that the expression of Cks1b in neural precursors is regulated by FGF2 in vitro, such that Cks1b is expressed at high levels in proliferating neural precursor cells and at very low to non-existent levels in non-dividing neural precursors. Taken together, these findings suggest that in vivo a similar situation is taking place, whereby in the presence of FGF, VZ progenitors maintain their proliferative state by up-regulating members of the SCF<sup>Skp2</sup> complex, specifically Cks1b, that promotes the targeted destruction of cell cycle inhibitors like p27<sup>KIP1</sup>. In the absence of FGF, levels of p27<sup>KIP1</sup> would be allowed to rise to the point that VZ progenitors would exit the cell cycle, proceeding down a pathway toward terminal differentiation.

In support of this, FGF2 has been shown to influence proliferation and cortical development in vivo both during embryogenesis (Vacccarino et al., 1999; Raballo et al. 2000) and in the adult mouse CNS (Martens et al., 2002). FGF2 and the receptors for FGFs are broadly expressed in the developing brain (Ernfors et al., 1990; Powell et al., 1991; Weise et al., 1993), including the neuroepithelium at the onset of neurogenesis (Vaccarino et al., 1999), and in the adult rat, FGF-receptor 1, which binds FGF2, is expressed in the ependymal and subependymal regions of the entire ventricular neuraxis of the CNS (Matsuo et al., 1994; Belluardo et al., 1997). Exposure of organotypic slices of embryonic spinal cord in chick, Wilcock et al. (2007) showed that the length of the cell cycle of neuroepithelial progenitors was accelerated after exposure to FGF, exhibiting cell cycle kinetics more characteristic of earlier symmetrically dividing precursors relative to later asymmetrically dividing precursors. This is corroborated by several in vitro examples that show that neural progenitors change their cell cycle kinetics in response to FGF stimulation (DeHamer et al., 1994; Lukaszewicz et al., 2002; Cavanagh et al., 1997; Caviness et al., 2003; Li and DiCicco-Bloom, 2004) and have been shown to up-regulate G1-phase cell cycle genes in response to FGF2 stimulation (Li and DiCicco-Bloom, 2004; Freeman et al. 1994). Thus, FGF2 may cause VZ progenitor cells to undergo additional cycles either by preventing their differentiation, or by actively promoting their progression from G1 into S, by activating G1 cyclin-cdk complexes, and alternatively, through the targeted destruction of cell cycle inhibitors like  $p27^{KIP1}$ .

### V. E2F and CDE/CHR sites in the Cks1b promoter act mostly as repressor elements in vitro

We find that two cis-regulatory motifs within the Cks1 promoter, E2F consensus site and CDE-CHR sites, control Cks1b transcription during the cell cycle. Our studies reveal that these sites have dual functions both individually as repressor elements in quiescent cells in G0/G1, and cooperate to activate the transcription of Cks1b during the G1/S phase transition in neural precursor cells.

It is well established that the E2F repressor E2F4 plays a prominent role in the repression of transcription of multiple cell cycle genes during G0/G1, while E2F activators, E2F1-3, are predominate at promoters of genes necessary for the G1/S phase transition (DeGregori et al. 1997; Attwooll et al., 2003; van den Heuvel and Dyson, 2008). Our findings are consistent with this model of E2F target gene regulation. We show that the endogenous Cks1b promoter is bound by the repressor E2F4 transcription factor in G0/G1, and reporter ChIP data in 293 cells showed that this interaction is mediated by the E2F consensus site identified in our in silico analyses. Moreover, we show that occupation of the Cks1b promoter by E2F4 continues from G0 into early G1 before releasing from the binding site. The absence of E2F4 at the Cks1b promoter in mid-to-late G1 could result either from re-localization to the cytoplasm, which is known to occur at this time, or by an alternative, unknown mechanism. This partitioning would in effect prevent E2F-4 from directly affecting transcription in S phase. We further show that E2F-4 is rapidly replaced by E2F-1, as cells enter mid-to-late G1 and induction of S phase.

Other cell cycle genes that are direct, physiological targets of E2F4 include cyclin A, Cdc2, Cdc25A, CDK2, two members of the E2F family (E2F2 and E2F3), and two members of the pRB family (pRB and p107), each of which has been shown to be E2F-responsive (Ren et al., 2002). The expression of the cyclin E gene is also regulated by E2F transcription factors, and has several E2F consensus sites within its promoter (Ohtani et al., 1995). The E2F consensus site we have identified within the Cks1b promoter is identical to one of the E2F sites described within the promoter of the cyclin E gene (Ohtani et al. 1995). That E2F site is also occupied by the E2F4/p130 complex exclusively in quiescent cells in G0/G1 and has been shown to act predominately as a repressor element, as mutation of this site resulted in elevated levels of promoter activity in quiescent cells without changes in activation (Le Cam et al. 1999).

## VI. Cks1b and Skp2 might be part of a regulon that is regulated by E2F transcription factors during cell cycle

It has been reported that Cks1b protein is differentially expressed during the cell cycle with the lowest level of expression in G0/G1 and progressively higher levels of expression in S-phase (Bashir et al. 2004). Rother et al. (2007) reported that Cks1b mRNA expression starts in late G1, increasing just prior to the rise of S-phase, topping out at 20 h following serum stimulation, coinciding with the highest proportion of cells have reached S/G2. We have shown that the expression of the Cks1b gene in neural precursor cells is regulated at the level of transcription and is cell cycle-dependent, with low expression in quiescent G0/G1 cells and high in precursors induced by FGF to proliferate. This response appears to be at least in part controlled through an E2F site in

the Cks1b promoter. Studies in fibroblast cell lines have shown that Skp2 expression is also regulated at the transcriptional level in response to mitogenic and anti-mitogenic signals (Carrano and Pagano, 2001), and in primary T lymphocytes, co-stimulation through TCR/CD3-plus-CD28 can directly regulate cell cycle progression by inducing transcription of Cks1 and Skp2 leading to ubiquitin-targeted degradation of  $p27^{KIP1}$ through the SCF<sup>Skp2</sup> ubiquitin ligase (Appleman et al., 2006). Interestingly, p27<sup>KIP1</sup> levels are elevated in Skp2-/- mice (Nakayama et al., 2000, 2001). The promoter region of human SKP2 contains three putative E2F binding sites (Zhang and Wang, 2005), and Appleman et al. (2006) have shown that mutagenesis of one of these E2F sites enhanced reporter activity, indicating that the SKP2 promoter acts as a node of integration for mitogenic and anti-mitogenic signals (Appleman et al., 2006). Since Skp2 requires Cks1b for the recognition of phosphorylated p27 (Ganoth et al., 2001; Spruck et al., 2001), we propose that Cks1b and Skp2 might be part of a regulon, falling under the regulation by the same regulatory protein, E2F, despite the genetic distance between the two genes. Control of these two genes by E2F, first by transcriptional repression in G1 followed by activation near the onset of S phase fits well with the known cell cycle activity of the SCF<sup>Skp2</sup> E3 ubiquitin ligase complex that is activated at S phase, directing the degradation of cell cycle substrates such as p27<sup>KIP1</sup>, following their phosphorylation by protein kinases (Nakayama and Nakayama, 2005).

## VII. The repression of Cks1b transcription in G0/G1 is conserved in rodents and humans

We find that E2F4 bound the Cks1b promoter at G0/G1 in both rat and human cell lines and in rat primary neural precursor cells. This conservation both between species and among tissues was unique to E2F4, and was not seen with E2F1 ChIP (discussed in greater detail below). Together with our luciferase and reporter ChIP data showing that the E2F site bound by E2F4 in the Cks1b promoter in G0/G1, our results suggest a highly conserved regulatory function for E2F4 in regulation of Cks1b transcription. Our finding is consistent with the findings of Conboy et al. (2007), who identified a set of E2F4target genes that were bound by E2F4 in both mouse and human cells. A key feature of the approximately fifty genes where E2F4 binding was conserved was their enrichment in cell cycle, proliferation, and DNA repair functions, with a preference for functionally relevant regulatory interactions important for maintaining cellular quiescence (Conboy et al., 2007). Given the high divergence in E2F4-target genes between rodent and human (Conboy et al., 2007; Odom et al., 2007), the E2F4 occupancy and functional regulation of the Cks1b gene at G0/G1 in all tissues and species we tested suggests that this particular mechanism for repression of Cks1b in G0/G1 is a significant one.

Coupling the information from the luciferase promoter-reporter and ChIP assays provided a time-point by time-point look at the functional consequence of E2F binding at the Cks1b promoter. This experimental approach might also provide a useful way of looking at RNA Pol II activity at the Cks1b promoter or at the promoter of other developmentally regulated gene. Stalled or paused RNA Pol II, allows a rapid transition into productive elongation (Muse et al., 2007), and preloaded RNA Pol II appears to
facilitate rapid and synchronous temporal and spatial changes in gene activity during development (Boettinger and Levine, 2009). Zeitlinger et al. (2007) performed comprehensive RNA Pol II ChIP-chip assays in Drosophila embryos and identified three distinct Pol II binding behaviors: active (uniform binding across the entire transcription unit), no binding, and stalled (binding at the transcription start site). The ~10% of genes that were stalled were highly enriched for developmental control genes, which were either repressed or poised for activation during later stages of embryogenesis (Zeitlinger et al., 2007). Our own experimental approach suggests that ChIP combined with luciferase promoter reporter data provides a unique way of overlaying gene activation data with information about promoter occupancy at identical timepoints in culture, thus presenting a clearer picture of the activity of genes harboring stalled or active RNA Pol II. In the case of Cks1b, this experimental approach could be used to distinguish the type of RNA Pol II present at the promoter in G0/G1 and throughout the cell cycle following FGF2 stimulation.

#### VIII.Mice deficient for E2F4 show modest effects during embryonic development

Given that the evolutionary conserved functional enrichment of E2F4-target genes are involved in several cellular processes including cell cycle, DNA repair, and DNA replication, one would predict that removal of E2F4 would have important implications for the proper gene expression of the genes bound by E2F4 in vivo. Instead, developmental recovery and survival to adulthood of E2F4-deficient mice corresponds with almost completely normal gene expression in multiple tissues, relative to their

phenotypically normal heterozygous counterparts (Conboy et al., 2007). Reports have indicated that the function of most tissues, even those directly impacted during development by absence of E2F4, eventually recovers. Mice lacking either E2F4 or E2F5 underwent normal embryonic development, and fibroblasts isolated from either knockout showed normal proliferation characteristics compared to wildtype (Lindeman et al. 1998; Rempel et al. 2000). The findings of Conboy et al. (2007) reveal that this recovery extends to the level of gene expression. It appears that the absence of E2F4 can have profound, yet transient, implications for tissue-specific transcriptional programs (Conboy et al., 2007). This recovery most likely depends on the overlapping roles that other members of the E2F family can play. For instance, it has been previously shown that E2F5 can largely compensate for the absence of E2F4 in vivo (Gaubatz et al., 2000). Combined inactivation of E2F4 and E2F5 resulted in late embryonic death, suggesting that the two E2Fs may functionally compensate for one another in development (Gaubatz et al. 2000). Interestingly, MEFs lacking E2F4 and E2F5 (but not single knockout E2F4-/- or E2F5-/- cells) were unable to quiesce even when presented with the p16<sup>INK4a</sup> cell cycle inhibitor (Gaubatz et al. 2000).

Whereas previous studies have revealed that E2F4-mediated repression is important during embryogenesis, few studies have looked at the role of E2F4 in the CNS. Based on our results in vitro, we would predict that loss of E2F4 would result in increased cellular proliferation among neural progenitors leading to larger CNS structures. The findings of Ruzhynsky et al. (2007), however, showed that E2F4-/- mice exhibited a dramatic loss of ventral telencephalic structures characterized by the absence or reduction of the lateral and medial ganglionic eminences. They further noted that in vivo E2F4 mutant embryos showed no difference in the number of proliferating cells in the dorsal or ventral ventricular zone regions of the telencephalon, nor did they see any disruption in the differentiation of early born neurons in the mantle region of the developing telencephalon (Ruzhynsky et al., 2007). Interestingly, when neuroepithelial cells were harvested from the telencephalon of E2F4-/- mice at E13.5 and plated at equal densities as neurospheres in vitro, cells derived from the telencephalon of E2F4-deficient embryos gave rise to 77% fewer primary neurospheres compared with wild type, indicative of a reduction in the number of sphere-forming cells in the mutant brains (Ruzhynsky et al., 2007). When they measured BrdU incorporation in monolayer cultures of neural progenitors in vitro, they found no difference in the proliferation index in cultures derived from wild-type and E2F4-deficient mouse embryos, indicating that E2F4 deficiency does not affect the rate of cell proliferation (Ruzhynsky et al., 2007). They found that Loss of E2F4 did not affect the rate of progenitor proliferation, and they proposed that instead, loss of telencephalic structures resulted from reduced size and selfrenewal capacity of the neural precursor pool in the telencephalon (Ruzhynsky et al., 2007). Nonetheless, their findings suggest that E2F4 deficiency does not influence the rate of cell division in neural progenitor cells or the timing of the onset of neuronal differentiation in the telencephalon in vivo.

One possible explanation as to why loss of E2F4 did not produce a more pronounced effect on neural progenitors in vivo could be because, in addition to regulating several cell cycle regulatory genes, including those encoding cyclins, CDKs, other E2Fs, and the pRb family, E2F4 is bound to genes involved in the G1 and G2 DNA damage checkpoints, DNA replication, and DNA repair. E2F4 also binds to genes that function to promote chromosome condensation and segregation as well as the spindle checkpoint (Ren et al. 2002). Therefore, loss of E2F4-mediated transcriptional regulation likely effects many target genes leading to the disruption in any number of cellular processes during CNS development that could mask or possibly compensate for loss of Cks1b repression in E2F4-deficient mice. Another possible explanation comes from further examination of the mechanism underlying the telencephalic defect found in E2F4 deficiency that revealed a dramatic reduction in Shh, a morphogen essential for the development of ventral structures (Ruzhynsky et al., 2007). Specifically, they found that E2F4 deficiency leads to aberrant activation of Shh brain enhancers (Ruzhynsky et al., 2007). Shh has been shown to promote cell division in neural progenitors in vitro (Weiss and Reynolds, 1992), perhaps losing Shh and possibly other important signals reduces the ability of neural progenitors to proliferate during early brain development in E2F4deficient mice.

# IX. In neural progenitors, E2F4 and p130 likely act as a repressor complex in G0/G1

E2F4 and p130 are the principal E2F and pRb family members bound to promoters in quiescent cells (Takahashi et al., 2000). In the absence of a mitogenic stimulus (or presence of some anti-proliferative signals), p130 is hypophosphorylated allowing it to bind in the cytoplasm with E2F4 and translocate to the nucleus where, in conjunction with chromatin remodeling proteins, they repress transcription of target

genes (Blais and Dynlacht, 2007). We have shown that in neural progenitors, the pocket protein p130 is present at the Cks1b promoter coincident with E2F4 occupancy at a time when Cks1b promoter activity is normally repressed. Embryos lacking p130 are smaller than wildtype mice, displaying a disorganized morphology and having fewer Isl-1/2expressing motor and sensory neurons as well as more apoptosis (LeCouter et al., 1998). In p130-/- mice, LeCouter et al. (1998) used PCNA expression as a marker for increased cellular proliferation to study the influence of loss of p130 on proliferation of neural progenitors and development of the brain. The numbers of PCNA-expressing cells were increased 2.1-fold in the telencephalon, 2.6-fold in the diencephalon, and 2.1-fold in the neural tube in E10.5 p130-/- embryos (LeCouter et al. 1998). In contrast to what has been reported for E2F4-/- mice in vivo, loss of p130 has a dramatic effect on the number of proliferating neural progenitors during development, suggesting that the role of E2F4/ p130 complex in inhibiting transcription of genes necessary for the G1/S transition may rely more on p130 than on E2F4. This makes sense because the the translocation of E2F4, and therefore re-location of the entire repressor complex to target gene promoters, depends on hypophosphorylated p130-mediated transport.

# X. The cell cycle-dependent element (CDE) and cell cycle genes homology region (CHR) also represses Cks1b in G0/G1

We also find that a tandem repressor element called cell cycle-dependent element (CDE) and cell cycle genes homology region (CHR) plays a role in the repression of the Cks1b gene expression in G0/G1 neural precursor cells. This tandem transcriptional repressor element is known to control other cell cycle-regulated genes commonly

expressed at the S/G2 transition like cyclin A, cdc2, and cdc25c genes (Zwicker et al. 1995). Like E2F repressor sites, mutation of these consensus sites in these gene promoters results in elevated levels of expression of a reporter gene in quiescent cells in G0/G1 cell cycle phase (Zwicker et al., 1995).

# XI. E2F and the CDE-CHR tandem element cooperate to regulate the activation of Cks1b transcription during the cell cycle

We show that in neural progenitor and 293 cells E2F1 is present at the Cks1b promoter in cycling cells, and that mutation of the E2F site alone did not significantly alter the induction of the Cks1b promoter activity in all cell types under study. It was only in the presence of the CDE-CHR mutation did we observe a delay in onset of activation, and this was only true of neural progenitors towards the end of the cell cycle. This suggests that the interaction between E2F1 and the Cks1b gene promoter in 293 cells is either non-functional or occurs at some other, non-canonical binding site. The fact that the Cks1b promoter contains a conserved CDE-CHR in close proximity to and just downstream of the E2F site whereas the cyclin E promoter does not would explain why our findings differ from those reported in Le Cam et al. (1999). There is no existing literature documenting an activator role for the CDE-CHR element; however, the promoters of several genes under CDE/CHR regulation also have E2F sites in close proximity to CHR or CDE-CHR sites, including B-myb (Le Cam and Watson, 1993; Catchpole et al. 2002), cdc2 (Shimizu et al. 1995), and cdc25C (Lucibello et al. 1995; Liu et al. 1997; Lucibello et al. 1997). It has been reported that the CDE of the cdc2 and cyclin A promoters can bind E2F complexes in vitro with low efficiency (Zwicker et al.

1995) other reports disagree with this (Liu et al. 1998). Moreover, the CDE-CHR sequence of the cdc25C promoter (Lucibello et al. 1997) is unable to bind E2Fs in vitro. These are consistent with those suggesting that the whole CDE-CHR module in the cdc2, cdc25C, and cyclin A promoters are occupied by an as yet to be identified factor, named CDF1, that is distinct from E2F complexes and which does not associate directly with pocket proteins (Haugwitz et al., 2002). It has also been shown that an unidentified factor called CHF (cyclin A CHR binding factor), may act independently of CDF- and E2F-activities and has been shown to bind only the CHR element of the cyclin A promoter (Liu et al. 1998). However, in the cases of CHF and CDF, protein species carrying these activities remain to be identified.

It has been shown that during the cell cycle, Cks1b protein is found in very low levels in G0/G1 and progressively higher levels in S-phase (Bashir et al. 2004). In Rother et al. (2007), it was reported that Cks1b mRNA expression starts in late G1, increasing just prior to the rise of S-phase, reaching a maximum expression around 20 hours after serum stimulation when the percentage of cells in S/G2 are at their highest levels. In light of these expression data, and considering that the majority of promoters carrying CDE-CHR elements have been shown to regulate cell cycle-dependent transcription of several G2/M genes (Zwicker et al. 1995; Lange-zu et al. 2000), it is tempting to speculate that disruption of both E2F and CDE-CHR sites might prolong the onset of induction because it confers a G2/M transcriptional profile on Cks1b, in other words, it causes Cks1b expression to peak later in the cell cycle.

# XII. p27<sup>KIP1</sup> is not required for neuronal differentiation during neurogenesis in the developing spinal cord

p27<sup>KIP1</sup> was originally identified as a regulator of the G1 phase of the cell cycle (Polyak et al., 1994; Toyoshima and Hunter, 1994). Consistent with this role, we find that ectopic expression of p27<sup>KIP1</sup> in ventricular zone cells in the developing spinal cord in ovo reduces the number of proliferating cells labeled by BrdU or expressing the G2-M marker phospho-H3 or S phase marker MPM2. This reduction in the number of proliferating neural progenitor cells is likely due to a prolonged G1 phase and eventual cell cycle withdrawal. It has been reported that transgenic mice overexpressing  $p27^{KIP1}$ in the CNS neuroepithelium show a lengthening of the G1 phase of the cell cycle in neural progenitor cells (Mitsuhashi et al., 2001). Although the mechanism by which p27KIP1 overexpression might prolong the time spent in G1 has yet to be established, it most likely reflects an alteration in the impact of the inhibitory effect of unbound p27<sup>KIP1</sup> on the kinase functions of the cyclin E-cdk2 complex required to drive G1 cells into the S phase (Mitsuhashi et al., 2001). Since the abundance of cyclin E is known to be transcriptionally regulated with each cell cycle (Sherr, 1994; Knoblich et al., 1994), overexpression of p27<sup>KIP1</sup> might prolong the time needed to synthesize cyclin E in amounts sufficient to exceed the threshold required for the G1/S phase transition (Mitsuhashi et al. 2001).

In addition to its cell cycle regulatory role, previous reports suggest that p27<sup>KIP1</sup> has a moonlighting function as a neural cell fate determinate. During gastrulation in frogs, p27<sup>Xic1</sup> was shown to promote the neuronal differentiation of undifferentiated, mitotically active cells within the neural plate in a function that was independent of its

ability to inhibit the cell cycle (Vernon et al. 2003). In the developing retina,  $p27^{Xic1}$ increases during retinal development, coincident with the differentiation of retinoblasts, and overexpression of p27<sup>Xic1</sup> induces retinal precursors to adopt a glial cell fate at the expense of other neuronal fates and that this function is distinct from its regulation of the cell cycle (Ohnuma et al., 1999). In the mammalian CNS, Nguyen et al. (2006) reported that forced expression of p27KIP1 led to premature neuronal differentiation in the VZ/SVZ of the developing mouse cerebral cortex. Specifically, they reported that by overexpressing p27<sup>KIP1</sup> in cortices of E14.5 mice, they were able to increase the number of cells expressing the neuronal markers HuC/D or BIII-tubulin while decreasing the number of cells expressing the progenitor marker nestin in the VZ/SVZ. Conversely, knockdown of p27KIP1 using p27KIP1-specific siRNA led to a significant reduction of the number of ßIII-tubulin-positive neurons and to an increase in the number of cells expressing the progenitor marker nestin (Nguyen et al. 2006). Our findings do not corroborate the findings of Nguyen et al. (2006). We show that cells within the ventricular zone of the developing vertebrate spinal cord expressing exogenous  $p27^{KIP1}$ do not prematurely express any of the neuronal-lineage markers BIII-tubulin, NF-M, HuD, and NeuN, indicating that forced expression of p27<sup>KIP1</sup> in neural progenitors is insufficient to drive them toward a particular cell fate.

Although our findings differ from those of Nguyen et al. (2006), our conclusion that p27<sup>KIP1</sup> is insufficient for neuronal differentiation is nonetheless in agreement with initial reports from p27<sup>KIP1</sup>-deficient mice that did not describe any neurological deficits nor any readily apparent CNS defects (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama

et al. 1996), as well as those of Tikoo et al. (1998), who showed that while ectopic expression of  $p27^{KIP1}$  in oligodendrocyte progenitors was sufficient to induce growth arrest in vitro even in the presence of mitogenic stimulation differentiation did not occur by default. In the adult SVZ, loss of p27<sup>KIP1</sup> resulted in an increased ability to generate new neurospheres in vitro, but the loss of p27<sup>KIP1</sup> did not not effect their potential to differentiate into any of the three neural cell types, O4-positive oligodendrocytes, GFAPpositive astrocytes, and Tuj1-positive neurons (Doetsch et al., 2002). Interestingly, Nguyen et al. (2006) noted that among the Cip/Kip CKIs, only p27<sup>KIP1</sup> was capable of promoting neuronal differentiation. This finding was not supported by Gui et al. (2007), who reported that, while p27KIP1 has the broadest expression in the developing spinal cord among the Cip/Kip genes, mice deficient for p27KIP1 showed normal neurogenesis in the developing neural tube, and that only p57<sup>KIP2</sup> knockout mice showed an increase in neurons in addition to excess proliferating cells. They also reported that neuronal differentiation in the developing spinal cord was able to proceed in the absence of all three Cip/Kip genes (Gui et al. 2007). The ability of p27<sup>KIP1</sup> to induce neuronal differentiation during neurogenesis may, therefore, be quite different in the developing cerebral cortex than in the developing spinal cord. In addition, Levine et al. (2000) showed that in the retinas of mice lacking p27<sup>KIP1</sup> the progeny of retinal progenitor cells expressed markers of both photoreceptors and Muller glia, suggesting that progenitors in p27<sup>KIP1</sup>-deficient retinas retained their multipotency and that p27<sup>KIP1</sup> was not required for cell fate specification or for the onset of differentiation, but rather to regulate the numbers of progenitors that generated those cell types (Levine et al., 2000). Thus, it is also

possible that p27<sup>KIP1</sup> may indirectly influence neural differentiation by disrupting normal cellular proliferation at the expense of differentiation during periods of histogenesis.

In the adult neurogenic zones, some evidence exists that support the idea that p27<sup>KIP1</sup> directly regulates cell cycle progression proliferation and indirectly influences It has been proposed that p27<sup>KIP1</sup> serves as an internal 'clock' cyto-differentiation. instructing proliferating cells when to stop dividing and exit the cell cycle (Durand et al. 1998; Casaccia-Bonnefil et al., 1999). In this capacity, p27<sup>KIP1</sup> is capable of influencing both cell cycle progression of progenitor cells and indirectly influences the fates of their sequentially generated progeny. The idea being that lengthening or shortening the proliferative capacity of progenitor cells by manipulating the levels of  $p27^{KIP1}$  protein would bias the production of one fate over a normally earlier or later fate (Durand et al., 1998; Ohnuma et al., 1999). Evidence supporting this idea stems from in vivo and in vitro work showing the expression of p27KIP1 gradually increases in accordance with the onset of neurogenesis and subsequent gliogenesis (Lee et al. 1996; Delalle et al. 1999) and ectopic expression of p27KIP1 in progenitor cells results in altered proliferative kinetics in response to mitogenic factors (Durand et al. 1998). Lee et al. (1996) showed by western blot analysis that levels of p27<sup>KIP1</sup> are initially low in extracts from E14 brain, near the onset of neurogenesis. However, levels of p27<sup>KIP1</sup> expression rise significantly by E16 and peak at E20 (nearly 10-fold versus E14 levels) coincident with late-stage neurogenesis, and expression of p27KIP1 remains at this elevated level in the adult brain (Lee et al. 1996). In vitro, oligodendrocyte precursor cells progressively accumulate amounts of p27<sup>KIP1</sup> protein as they proliferate (Durand et al., 1997). In the adult SVZ,

loss of p27<sup>KIP1</sup> causes neural progenitor cells to undergo additional rounds of cell division at the expense of lineage progression (Doetsch et al. 2002). In that study, it was shown that adult SVZ neural progenitors from p27<sup>KIP1</sup>-/- mice did eventually progress into proliferating migratory neuroblasts, suggesting that other cell cycle inhibitors might be involved in commitment of progenitors to a particular lineage and eventually terminal differentiation (Doetsch et al. 2002).

We found that when we ectopically expressed p27KIP1 in VZ progenitors in the developing spinal cord in ovo, a number of p27<sup>KIP1</sup>-positive cells were stuck in the VZ, whereas other p27<sup>KIP1</sup>-positive cells were able to migrate away from the VZ into the mantle zone. The neural progenitors residing in the VZ undergo interkinetc nuclear migration, passing through M phase and eventually dividing at the ventricular surface, and generate new neurons after withdrawing from the cell cycle. In contrast to the VZ cells, the intermediate progenitor cells of the SVZ in the cerebral cortex are essentially stationary, dividing at the same place where they pass through S phase. Newly generated post-mitotic neurons then migrate away from the VZ, or SVZ in the cerebral cortex. One possible explanation for the apparent lack of motility of some VZ progenitors and not others might be that forced expression of p27<sup>KIP1</sup> and cell cycle exit somehow disrupts the cytoskeletal machinery that orchestrates the location of the nucleus within the pseudostratified neuroepithelium rendering some progenitors motile and others not, depending on their proximity to the ventricular surface, for example. The cytoskeletal mechanisms regulating the localization of the nucleus within neuroepithelial cells during the course of the cell cycle and interkinetic nuclear migration is not fully understood.

Another potential explanation would be that forced expression of p27KIP1 in VZ progenitors perhaps prevents some VZ progenitors from accessing the machinery necessary for successful migration out of the VZ. Neuroepithelial cells are highly polarized and elongated, with a specific organization of proteins at either apical or basal contact (Huttner and Brand, 1997; Wodarz and Huttner, 2003). For example, the transmembrane protein prominin-1 (CD133) is found exclusively in the apical plasma membrane (Aaku-Saraste et al., 1996; Zhadanov et al., 1999; Manabe et al., 2002), tight junctions and adherens junctions are present at the most apical end of the lateral plasma membrane (Wodarz and Huttner, 2003), and receptors for basal lamina proteins such as integrin alpha-6 are concentrated in the basal plasma membrane, which contacts the basal lamina (Wodarz and Huttner, 2003). The motility of neuroepithelial cells in the developing spinal cord, therefore, might depend not only on the appropriately timed expression of p27<sup>KIP1</sup> and cell cycle exit, but on the appropriate positioning of the nucleus and/or access to apical or basal machinery before newly generated neurons can successfully navigate their way from their origins in the VZ to their final destination in the mantle zone.

## XIII.p27<sup>KIP1</sup> might act as a strong brake in post-mitotic neurons preventing their reentry into the cell cycle and procuring their survival

In the rat brain, p27<sup>KIP1</sup> levels rise over the course of neurogenesis and remain elevated into adulthood (Lee et al., 1996). This suggests that p27<sup>KIP1</sup> might play an important role in post-mitotic neurons after the completion of neurogenesis. A number of studies have described a link between cell cycle regulation and neuronal cell death, and evidence suggests that cell cycle re-entry represents an important mechanism of neuronal apoptosis (Liu and Greene, 2001). Neuronal cell death in vertebrates is both a normal developmental process and the detrimental outcome of nervous system injury or neurodegenerative disease. Cell cycle regulators, especially those important for the G1-S phase transition, have been found to be deregulated in diseased neuronal tissue and apoptotic neurons (Liu and Greene, 2001). For example, E2F1 is up-regulated in neurons subjected to apoptotic stimuli in vitro (O'Hare et al. 2000; Hou et al. 2001; Trinh et al. 2001; Verdaguer et al. 2002; Boutillier et al. 2003) and in vivo (Osuga et al. 2000), while neurons cultured from E2F1-deficient mice show some protective qualities in response to apoptotic stimuli (Giovanni et al. 2000; Hou et al. 2000; O'Hare et al. 2000; Hou et al. 2001; Gendron et al. 2001). Promoters of B- and C-myb genes, transcription factors with proapoptotic activity, contain E2F consensus sites (Oh and Reddy, 1999; Catchpole et al. 2002), are up-regulated in cultured neurons subjected to apoptotic stimuli that is mediated by E2F repression by E2F4-p130 (Estus et al. 1994; Liu and Greene, 2001, 2004). In viable neurons in G0, E2F4-p130 complexes have been found at the promoters of cell cycle genes necessary for G1-S phase transition, and this complex probably involves chromatin modifiers as HDAC1, Suv39H1 and HP1alpha (Liu and Greene, 2001; Liu et al., 2005; Panteleeva et al., 2007). In terminally differentiated neurons, the regulation of these factors is crucial, as inappropriate re-activation of cell cycle genes leads to neuronal apoptosis and progression of neurodegenerative diseases (Herrup et al., 2004). Thus, one potential role for p27<sup>KIP1</sup>, and perhaps other CKIs as well, in adult post-mitotic neurons is to actively prevent cell cycle re-entry.

Loss of endogenous CKIs might permit elevated Cdk activity and neuron death. Consistent with this idea, levels of p27Kip1 declined in cultured cerebellar granule neurons after potassium-induced repolarization (Padmanabhan et al. 1999; Martin-Romero et al. 2000) and in cultured neocortical neurons subjected to oxygen-glucose deprivation using a mild ishemia model (Katchanov et al. 2001). The latter study concluded that loss of endogenous CKIs was a likely trigger for re-entry of post-mitotic neurons into the cell cycle, noting that neurons that survived the ischemia did not show any loss of CKI proteins (Katchanov et al., 2001). Additionally, sections of brains taken from mice lacking both p19<sup>INK4d</sup> and p27<sup>KIP1</sup> showed cells expressing neuronal markers in the adult brain that were also immunopositive for BrdU, suggesting that these postmitotic neuronal populations had re-entered the cell cycle (Zindy et al. 1999). Such findings support the idea that CKIs play an important role in suppressing cdk activity in post-mitotic neurons, and that they function to prevent cell cycle re-entry as well as cdkdependent apoptosis. Although we did not test this idea directly in this study, our data seem to suggest that in post-mitotic neurons p27<sup>KIP1</sup> serves, at least in part, to prevent relapse into the cell cycle, which would result in neuronal cell death.

#### CONCLUSION

In this study, we investigated the role of p27KIP1 in regulating the cell cycle progression and differentiation of neural precursor cells during neurogenesis in the developing vertebrate CNS. We show that neural progenitor cells residing in the ventricular zone of the developing cerebral cortex and spinal cord actively suppress the expression of p27<sup>KIP1</sup> protein, and that in vitro, neural progenitors up-regulate the expression of the SCF<sup>Skp2</sup> component Cks1b in response to FGF2 stimulation, coinciding with a decrease in p27<sup>KIP1</sup> protein expression, presumably through ubiquitin-mediated proteasomal degradation. We also show that the subcellular localization of p27KIP1 in neurons is variable depending on developmental stage and axial level of the developing spinal cord. We further show that Cks1b promoter activity is induced prior to S phase in cycling neural progenitor cells, and that two cis-regulatory elements in the Cks1b gene promoter, an E2F consensus site and CDE-CHR element, are involved in the regulatory program(s) controlling the cell cycle-dependent expression of the Cks1b gene. Ectopic expression of p27<sup>KIP1</sup> in ovo reduced the number of proliferating cells in the early spinal cord diminishing its overall size and shape, suggesting that p27<sup>KIP1</sup> does play some role in cell cycle progression of neural progenitors. Taken together, these findings suggest that neural precursor cells maintain their proliferative state by suppressing p27<sup>KIP1</sup> protein levels in response to mitogenic factors by up-regulating members of the SCF<sup>Skp2</sup> ubiquitin ligase complex, targeting p27<sup>KIP1</sup> for proteasomal degradation and facilitating cell cycle progression. Thus, mitogenic factors would promote precursor cell proliferation by both activating positive cell cycle regulators like G1-type cyclins and cdks, as well as by

delaying the onset of terminal mitosis and differentiation by downregulation of negative regulators like p27<sup>KIP1</sup>. Evidence also suggests that p27<sup>KIP1</sup> may play another role in preventing neurons from returning to the cell cycle and preserving neuronal cell survival, but this needs to be addressed directly.

### **METHODS**

#### Isolation and culture of rat embryonic neural precursor cells (NPCs)

Neural precursor cells were prepared as described by Johe et al. (1996) and Whittemore et al. (1999). Rodent dams and pups were sacrificed using protocols approved by the University of Miami, Animal Care and Use Committee. Sprague-Dawley embryonic rat cerebral cortices (gestation day 14; day of conception is day 0; Harlan) were dissected in Ca2+- and Mg2+-free Hank's buffered saline solution (HBSS) and dissociated by brief mechanical trituration with a P1000. The cell suspension was allowed to settle and supernatant containing single cells was diluted in serum-free growth medium N2 (Bottenstein and Sato, 1979) and assessed for viability with trypan blue. Routinely approximately 80% viable cells were obtained. 6 x 10<sup>6</sup> were plated per 10cm plastic tissue culture plate that was pre-coated with 15 ug/ml of poly-L-ornithine (PO) and 1ug/ml of bovine plasma fibronectin (FBN). Cells were grown at 37C in a 5% CO2 water-jacketed incubator in N2 supplemented with 10 ng/ml FGF2 (R&D systems). FGF2 was replenished daily and medium changed every 2 days. Cells were passaged approximately 40-50% confluence (4 days after plating) by briefly incubating them in HBSS (Ca2+- and Mg2+-free) and dislodging the cells with a lifter. Where indicated, cultures were synchronized by withdrawing FGF2 form the culture media for 48 hours prior to re-application of 20 ng/ml FGF2.

#### HEK 293 and Rat2 Cell Line Culture

HEK 293 and Rat2 cells were obtained from ATCC. Cells were grown in DMEM containing 10% fetal bovine serum (FBS) and were made quiescent by removal of serum for 48 hours. Where indicated, cells were stimulated to enter the cell cycle by adding 20% FBS to the medium. Cell cycle position was monitored by propidium iodide staining and flow cytometry.

#### **DNA Content Analysis**

P2 Neural progenitors were synchronized by mitogen deprivation for 48 hours after which they were stimulated with fresh N2 media supplemented with 20 ng/ml FGF2 (R&D systems). Cells were lifted using a 1:1 solution of N2 media and Trypsin and passed through a 0.2 um filter. NPCs were counted and approximately 1x10<sup>6</sup> cells were fixed in 70% ethanol for flow cytometry analysis. HEK 293 and Rat2 cells were synchronized by serum deprivation for 48 hours prior to re-application of 20% FBS. Cells were lifted using 0.25% Trypsin-EDTA and passed through a 0.2 um filter. Cells were counted and approximately 1x10<sup>6</sup> cells were fixed in 70% ethanol for flow cytometry analysis. Fixed cells were fixed in 70% ethanol for flow cytometry and passed through a 0.2 um filter. Cells were counted and approximately 1x10<sup>6</sup> cells were fixed in 70% ethanol for flow cytometry analysis. Fixed cells were stored at -20 for a maximum of one week. For staining with propidium iodide, fixed cells were pelleted by centrifugation for 5 min at 2500 rpm, rinsed twice with PBS, then stained with solution of PI (1mg/ml) RNase A (10mg/ml) and Ca2+Mg2+free PBS. Stained cells were transferred to a 1.5ml round bottom tube and analyzed by flow cytometry.

#### **Reverse transcription-PCR**

Total RNA was prepared from neural progenitors cells in the presence or absence of FGF as indicated, or from whole E14 rat embryo, using Trizol ® reagent (GIBCO) as directed by the manufacteurer's protocol, which is based on early protocols from Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. 162, 156.; Chomcyzinski, P. (1993) Biotechniques 15, 532. First-Strand cDNA synthesis was performed using Superscript III TM Reverse Transcriptase (Invitrogen) with 10ug of total RNA and random hexamers (Invitrogen). PCR was performed using the FailSafe PCR kit (Epicentre). 1ul of cDNA was used per PCR reaction in a 25-ul volume with 200mM of each of the following gene-specific primers: rat p27Kip1 Forward (5'-ttgcgcaattaggtttttcc-3') Reverse (5'-ccagagttttgcccagtgtt-3'); rat Cks1b Forward (5'-gaattcgagtaccggcatgt-3') Reverse (5'-cttctgacggatggcaaaat-3'); rat E2F1 Forward (5'-gtgcagaaacgacgcatcta-3') Reverse (5'-aatcccctctgcactttcct-3'); rat E2F2 Forward (5'-agcagcccccagctctgcccg-3') Reverse (5'-ccctgcctacccactggatgt-3'); rat E2F3 Forward (5'-atggacagctccggcactcac-3') Reverse (5'-atattcccattgtggtcttgg-3'); rat E2F4 Forward (5'-atggcggaggccgggccaca-3') Reverse (5'-cctccaggctggtgcccgatg-3'); rat E2F5 Forward (5'-Forward (5'-agcactggggagaaaggatt-3') Reverse (5'-tacagggtattgcgagcaga-3'). Primers were designed using the Primer3/BLAST software available at the NCBI website. The PCR protocol was as follows: Denature step 98C 1 min., Anneal 62C for 30 sec, Extend 72C for 1 min, repeat for 32 cycles. 1Kb DNA ladder (Invitrogen) was used as DNA molecular weight standard.

#### **Commercial Antibodies**

The following commercial antibodies were used for Western blots analysis: anti-Cks1b rabbit polyclonal antibody (SC-6238); anti-Gapdh mouse monoclonal antibody (Ambion). The following commercially available antibodies were used for ChIPs: antip130 Rbl2 rabbit polyclonal antibody (SC-317X); anti-E2F4 rabbit polyclonal antibody (SC-1082X); anti-E2F1 rabbit polyclonal antibody (SC-193X) were obtained from Santa Cruz Biotechnologies. Normal rabbit IgG negative control (Millipore). The following commercially available antibodies were used for immunostaining: monoclonal anti-p27 antibody (BD Transduction Labs); polyclonal anit-Tbr-1 antibody (Millipore); monoclonal anti-betaIIItubulin (Tuj1) (Molecular Probes) ; polyclonal anti-neurofilament medium (NF-M) (Chemicon); conjugate anti-IB4 (Invitrogen); monoclonal anti-HuD antibody (Molecular Probes); monoclonal anti-NeuN antibody (Chemicon); polyclonal anti-MCM2 antibody (Bethyl); anti-MPM2 ; polyclonal anti-phospho-Histone H3 (Ser10), clone MC463 (Millipore).

#### Immunocytochemistry

Cells fixed with 4% para-formaldehyde (PFA) for 15 min were briefly permeabilized with 0.2% TritonX-100, blocked with 10% normal goat serum (NGS), incubated with primary antibody for 45 min, washed and incubated with secondary for 30

min, stained with DAPI (0.3 ug/ml) for 10 min at room temperature, and mounted with Citifluor (2.5% 1,4 diazabicyclo[2,2,2]octane (DABCO), 90% glycerol). All washes were with phosphate-buffered saline (PBS) three times for 5 min each.

#### Immunohistochemistry

Prior to staining, tissue was fixed with ice cold 4% paraformaldehyde (in 0.1M phosphate buffer, pH 7.2) for 45 min to 2 hours depending on the age of the embryo. After fixation, tissue was rinsed extensively with ice cold PBS for up to one hour. Tissue was then transferred to cold 30% sucrose (in 0.1M phosphate buffer, pH 7.2) and allowed to equilibrate overnight at 4C. Tissue was then embedded in mounting matrix and sections were collected at 20 um using a cryostat. Sections were permebealized with 0.4% TritonX-100 for 10min, blocked with 5% serum and incubated with primary antibody overnight at 4C. Secondary antibody incubated for 45min, DAPI for 10 min and covered with VECTASHIELD mounting medium.

#### Immunoblotting

Lysates were prepared and immunoblotting performed according to standard methods. As a positive control, cells were transfected with a commercially available recombinant Cks1b. Antibodies were titrated and were used at optimal dilutions to probe blots. Specific bands were detected by enhanced chemiluminescence (GE Biosciences). Blots were reprobed for Gapdh, which served as a control for gel loading. Benchmark prestained ladder (GIBCO) was used as molecular weight standards.

#### In Situ Hybridization

RNA probes were generated by PCR using the following primers: Cdkn1b Forward (5'-GAAATCTCTTCGGCCCGGTC-3')(608-627) Reverse (5'-TTCGGAGCTGTTTACGTCTGG-3')(1104-1124) Cks1 Forward (5'-CTCACTCCCTGCCATCTTTCTG-3')(362-383) Reverse (5'-CAAGGCTTGTAACATGGAAGAG-3')(709-730) Skp2 Forward (5'-ATTCTGTCCGAGTGCTCCAAG-3')(600-621) Reverse (5'-AGCGAGAGGTGTTGGAGGTAG-3')(1005-1025) Ngn2 Forward (5'-TTCGTCAAATCTGAGACTCTGG-3')(4-26) Reverse (5'-GAGCGCCCAGATGTAATTGTG-3')(472-492) Gap43 Forward (5'-GCTCAAAGGCGAGAAGAAGG-3')(150-169) Reverse (5'-TCATCCTGTCGGGCACTTTC-3')(625-644). Numbers in parentheses correspond to primer location relative to the transcription start site. The sections were fixed in 4 paraformaldehyde in 0.1 M phosphate buffer (PB) for 20 minutes, washed in PB, treated with 10ug/ml proteinase K in 50 mM Tris-HCl and 5 mM EDTA for 10 minutes, and then returned to the fixative solution. After washing in distilled water, the sections were acetylated with 0.25 acetic anhydride in 0.1 M triethanolamine, rinsed with PB, dehydrated in an ascending ethanol series (70%, 95% and 100%), defatted in chloroform, rinsed in ethanol, and then air dried. DIG-labeled antisense-RNA probes were prepared by in vitro transcription of each cDNA using SP6 or T7 RNA polymerase (Promega, Madison, WI, USA) and the DIG RNA labeling kit(Roche Diagnostic, Mannheim, Germany).

For hybridization, labeled probes in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 10 mM PB, 10 dextran sulfate, 1xDenhardt's solution, 0.2 sarcosyl and 500 mg/ml yeast transfer RNA) were denatured for 5 mimutes, quenched on ice and placed on sections. Hybridization was performed overnight in a humid chamber at 65°C. Hybridized sections were soaked in 5X SSC for 10 min at 70°C, incubated in 20X SSC for 2 hours at 70°C, and then washed with wash buffer(0.3 M NaCl, 10 mM Tris-HCl ph7.0, 5mM EDTA and 50% Formamide). After rinsing sections in Buffer1 (100mM Tris,ph7.5 and 150mM NaCl) for 5 min, they were treated with blocking buffer (10% normal goat serum,0.2% triton and 24mg/ml levapamizole in buffer1) for 30 minutes at room temperature and incubated in buffer1 containing 1:1000 of anti-DIG antibody (Roche) with 1% normal goat serum for 2 hours at room temperature, and washed for 3X5 min in buffer1. Sections were then briefly washed in buffer2 (100mM Tris ph9.5, 100mM NaCl and 50mM MgCl2), and incubated in buffer2 containing NBT/BCIP stock solution overnight. Finally development reaction was stopped in PBS.

#### **Chromatin Immunoprecipitation (ChIP) Assay**

Chromatin Immunoprecipitation (ChIP) assays were performed using the EZ ChIP Kit (Millipore) according to the manufacturer's instructions, with the following modifications. HEK 293 cells and Rat2 cells were synchronized by serum starvation and cells were harvested for ChIP immediately before re-application of serum (time point 0) and 18 hours after stimulation, time points that correspond to G0/G1 and S phase,

respectively. For ChIP with HEK 293 cells, a variety of time points were taken after stimulation as indicated. P2 neural progenitors were plated at a density of  $1 \times 10^{5}/15$  cm dish, and were synchronized by mitogen withdrawal and re-application. NPCs were harvested just prior to stimulation with bFGF (20ng/ml) and 18 hrs post-stimulation. The native protein-DNA complexes were cross-linked by treatment with 1% formaldehyde (from fresh 18.5% formaldehyde) for 10 minutes. Cross-linked genomic DNA was sheared using a Branson Sonifier ® S-250A analog ultrasonic processor to yield to produce fragments of 500bp in size. Briefly, equal aliquots of sheared chromatin were subjected to immunoprecipitation with polyclonal anti-E2F4 (SC-1802X), anti-E2F1 (SC-193X), anti-p130 (SC-317X) or a rabbit mock IgG antibodies (Millipore). DNA associated with immunoprecipitates was used as a template for PCR analysis with genespecific primers: Human CKS1B Forward (5'-GGCGGGACTTAGACAACAAG-3') Reverse (5'-TAGTGAGGCCGGAAGTGAGT-3')(242 bp) Human β-Actin Forward (5'-TGACAAGGACAGGGTCTTCC-3') Reverse (5'-CACCGTCCGTTGTATGTCTG-3') (169 bp); Rat Cks1b Forward (5'-GCACTGCACTCCATTTACGA-3') Reverse (5'-AGGCGGGACACCTCTACTTT-3')(128 bp); Rat Cdc6 Forward (5'-CCCTAATGGGAAGCACTTGA-3')(221 bp) Reverse (5'-CTGAGCTGGGATAGCCAGAG-3'). In the Rat2 ChIP experiments, we controlled for non-specific immunoenrichment of chromatin by the E2F antibodies, the same DNA was used as a template for PCR analysis with primers specific for a sequence roughly 2000 bp upstream of the rat Cks1b gene translation initiation start codon. Primers for rat Cks1b 2kb Forward (5'-CTAATTCGGGTGGTGCATCT-3') Reverse (5'-

AGGGGTGGCCAGATCTCTAT-3')(155 bp). Both qualitative end-point PCR (Epicentre FailSafe PCR Kit) and real-time PCR were performed (Epicentre GREEN Failsafe Real-time PCR Kit). For all real-time PCR analyses, an Eppendorf Mastercycler (a) ep realplex plate reader and associated realplex real-time PCR software was used. Threshold values (CT) were calculated and used to calculate the proportion of input enriched for in ChIP %Input=2(-deltaCt[normalized ChIP]).

#### **Strategy for Cks1b Promoter Reporter Constructs**

Primers used to clone the promoter fragments of the Cks1b gene promoter are as follows:1 kb Forward (5'-TGTGTTTGAAAGCGAGGCCAAGGATCCAAGCTT-3') Reverse (5'-GCTAGCATCGATCACTTCCGGCCTCACTAACCC-3'); 338 bp Forward (5'-GCTAGCATCGATCACTTCCGGCCTCACTAACCC-3') Reverse (5'-AAGCTTGGATCCTGGCCTCGCTTTCAAACACA-3'); 478 bp Forward (5'-GCTAGCATCGATCCCAGACAAACTTTATTACAC-3') Reverse (5'-AAGCTTGGATCCCCGGTACTCGAATCCTCGTC-3'). Mutations were inserted into the 338 bp wildtype promoter fragment using the overlap extension site-directed mutagenesis PCR method. Primers for the E2Fx mutant are as follows: Outer Forward (5'-GCTAGCATCGATCACTTCCGGCCTCACTAACCC-3') Outer Reverse (5'-AAGCTTGGATCCTTGGCCTCGCTTTCAAACACA-3') Inner Forward (5'-GCTAGCATCGATCACTTCCGGCCTCACTAACCC-3') Outer Reverse (5'-GCCGAAGGCTCTACAGCTCCA-3') The mutation to the CDE-CHR elements were made simultaneously using the following primers: CDE-CHRx Inner pair Forward (5'-

ACCAA	TAGGA	AGTG	CATTA	GGTGT	GTGCA	TAAGC	GAGG	CCAAA	GAG-3	')
R	e	V	e	r	S	e	(	5	,	-
CTCTTT	GGCCT	CGCTTA	ATGCAC	CACACO	CTAATG	CACTTO	CCTATT	'GGT-3').	Finall	y,
The double mutation (Dblx) was created by inserting the E2F mutation into the CDE-										
CHRx mutant promoter fragment. Promoter fragments were subcloned into the pGL4.17										
plasmid (Promega) at the NheI/HinDIII sites and fused with a luciferase reporter gene.										
Fusion co	onstructs	were the	n subclo	ned into	a promo	terless le	ntiviral v	vector (pL	entiMP2	2)
at the Cla	I/XbaI s	ites for pi	oductior	n of lentiv	virus for	luciferase	e assays i	in Rat2 an	d NPCs	•

#### **Dual luciferase Assay**

HEK 293 cells were transfected using Lipofectamine (Invitrogen) in serum rich media and 5 hours later serum was removed (0%FBS) for a period of 48 hours in order to synchronize the cells. After re-application of serum (20%FBS) cells lysed at indicated time points that correspond to the phases of the cell cycle. Rat2 cells were infected and 24 hours later split 1:5, 24 hours later cells were synchronized by serum deprivation (0%FBS) for a period of 48 hours. After that 48 hours, cells were stimulated to reenter the cell cycle by re-applying 20%FBS. Cells were harvested at the indicated time points. P1 neural progenitor cells were maintained as neurospheres in the presence of bFGF (10 ng/ml) at a density of 5x10^5/6cm dish at least 24 hours prior to lentiviral infection. Neurospheres were infected with either a wildtype or mutant Cks1b promoter luciferase reporter, as well as a constitutively active humanized renilla luciferase reporter, at a ratio of 10:2. Twenty-four hours following infection, NPCs were plated onto 10-cm dishes

coated with poly-ornithine and fibronectin to be grown as a monolayer. Three hours later, N2 media was replaced with N2 media minus bFGF. NPCs were mitogen starved for 48 hours before stimulating with bFGF (20 ng/ml) in order to synchronize them. NPCs were then harvested just prior to mitogen stimulation, as well as 6, 12, 24, and 36 hours post-stimulation. The lysis buffer used to harvest HEK293, Rat2, and NPCs was purchased from Promega and made fresh for every harvest. Lysates were kept at -80 prior to taking a luciferase measurement. For each measurement, 20ul of lysate was transferred to a well within a 96-well plate and treated with reagents from Promega's Dual-Glo luciferase assay kit per manufacturer's instructions.



ffluc2 MOI = 16,000 (80E+08 Viral particles / 5E+05 cells)

hRluc MOI = 3,200 (16E+08 Viral particles / 5E+05 cells)

**Figure 36**. Timeline illustrating protocol for transducing Rat2 cells. 24 hours after transduction, infected Rat2 cells were split 1:5 and the next day serum was removed (0%FBS) for 48 hours to synchronize cells. After 2 days, serum was re-applied

(20%FBS) and lysates were gathered at time point zero and at time points corresponding to the cell cycle phases. Multiplicity of infection (MOI), or number the number of infectious particles over the number of cells in culture, was kept constant throughout the experiments.



**Figure 37**: Timeline illustrating protocol for transducing NPCs. NPCs were passaged at least once after harvest from E14 rat telenephalon and were maintained in FGF2 (10 ng/ml) for 3 days. NPCs were then lifted and plated on uncoated dishes to encourage formation of floating neurospheres that were infected 24 hours later (micrograph shows a representative group of neurospheres). Multiplicity of infection (MOI), or number the number of infectious particles over the number of cells in culture, was kept constant throughout the experiments. 3 hours post-infection cells were plated onto dishes that were pre-coated with fibronectin and poly-ornithine. Cells were then synchronized as monolayers by removing FGF2 from the media for 48 hours (micrograph shows an example of NPCs grown as a monolayer on fibronectin dishes during synchronization). After 48 hours synchronized NPCs were then stimulated to reenter the cell cycle by reapplying FGF2 (20ng/ml). Lysates for dual luciferase assay were taken at time points coinciding with the phases of the cell cycle.

#### **Reporter ChIP Assay**

HEK 293 cells were plated at a density of 2x10<sup>6</sup> cells on a 10-cm plastic tissue culture dish. Cells were transfected with 12 ug of either wildtype or E2Fx mutant Cks1b promoter reporters. Twenty-four hours after transfection cells were split 1:5. Synchronized cells were processed for ChIP after 48 hours of serum deprivation. Primers specific for the exogenous Cks1b promoter reporter were made: Forward (5'-AGCCAATCGAGGACTTCAAA-3') Reverse (5'-TCGATATGTGCGTCGGTAAA-3') (243 bp). Both qualitative and real-time PCR were performed on immuno-enriched DNA as described above for normal ChIP.

#### Ectopic Expression of p27Kip1 by In Ovo Electroporation

Full length chicken p27Kip1 was cloned by PCR and ectopically expressed in chick embryos. Electroporations were performed as described previously (Pekarik et al., 2003). Briefly, chicken embryos (Charles River, SPAFAS) were injected at E2 to E2.5 [Hamburger and Hamilton stages (HH) 10-12] and dissected 48 to 72 hours later. DNA was injected into the lumen of the central canal at a final concentration of 500 ng/ul for each plasmid and electroporation was performed using a BTX Electro Square Porator ECM 830 (Genetronics) configured to deliver 5x50 msec pulses of 30 V across the embryo through a pair of gold-plated electrodes 5 mm in length, 0.5 mm diameter (Genetrodes Model 512, Genetronics, Inc.). Embryos were allowed to incubate at 37 celsius for 48-72 hours prior to dissection and staining.

### Bromodeoxyuridine (BrdU) Proliferation Assay

A total of 5 ug/ml BrdU was injected into the lumen of the neural tube of chick embryos 30 minutes before harvesting. BrdU detection was performed on sections by treatment with 2N HCl for 30 minutes, 0.1M NaBorate (pH 8.5) and incubation with anti-BrdU antibody.

### **TUNEL** Assay

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labelling (TUNEL) on cryosections was performed by using the In Situ Cell Death Detection kit (Roche Diagnostics) per manufacturer's instructions.

#### **Statistical Analysis**

Statistical significance for dual luciferase assays was determined by Two-way ANOVA followed by Newman-Keuls multiple comparison test using the PRISM5 statistical software package from GraphPad. Difference was considered significant at p<0.05. Experiments were performed at least 3 times in duplicates or triplicates and error bars on all histograms represent the mean values +/- SEM.

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