MOLECULAR MECHANISMS REGULATING FATE DETERMINATION OF CEREBRAL CORTEX PRECURSORS

by

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THESIS ABSTRACT

During development of the mammalian nervous system, neural stem cells generate neurons first and glia second, thereby allowing the initial establishment of neuronal circuitry, and subsequent matching of glial numbers and position to that circuitry. Multiple molecular mechanisms act in concert to control neural precursor expansion prior to neurogenesis, and to allow for an exponential generation of neurons while ensuring the maintenance of sufficient precursors to produce later-born neurons, glial cells and adult neural stem cells. Throughout cortical development, these processes are regulated in part by the precursors’ environment as well as intrinsic changes in precursors and their modes of division, which regulate the fate of daughter cells and the balance between self-renewal and differentiation. In the first part of this thesis, the protein tyrosine phosphatase SHP-2 was identified as a novel signaling protein that regulates the neurogenic to gliogenic switch by potentiating neurogenic signals and suppressing gliogenic signals until the appropriate developmental time point for astrogenesis, providing one mechanism whereby precursors integrate conflicting environmental cues. A Noonan Syndrome (NS)-associated activated SHP-2 mutation causes perturbations in neural cell genesis, which may contribute to the mild mental retardation and learning disabilities observed in NS patients. In the second part of this thesis, a novel Rho-regulatory pathway which includes the Rho-GEF Lfc and its negative regulator Tctex-1 were also found to regulate neurogenesis, potentially by directing mitotic spindle orientation during precursor divisions, thereby regulating the symmetric and asymmetric nature of radial precursor divisions.
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LIST OF ABBREVIATIONS

A-C/EBP, acidic C/EBP
AchR, acetylcholine receptor
AGS3, activator of g-protein signaling 3
AJ, adherens junction
aPKC, atypical protein kinase c
ASD, autistic spectrum disorder
ASPM, Abnormal Spindle-like, Microcephaly-associated
BDNF, brain-derived neurotrophic factor
bHLH, basic helix loop helix
BLBP, brain lipid binding protein
BMP, bone morphogenetic protein
BMPR, bone morphogenetic protein receptor
BrdU, Bromodeoxyuridine
bZIP, basic region-leucine zipper
C/A-C/EBP, constitutively active C/EBP
C/EBP, CCAAT/Enhancer-binding protein
Ca2+, calcium
CBP, CREB binding protein
Cdc42, cell division cycle 42
CDK, cyclin-dependent kinase
CFC, cardio-facial-cutaneous
CKI, cyclin-dependent kinase inhibitor
Cl-, chloride
CLC, cardiotrophin-like cytokine
CNS, central nervous system
CNTF, ciliary neurotrophic factor
CP, cortical precursor
CT-1, cardiotrophin-1
C-terminal, carboxy-terminal
DCLK, doublecortin-like kinase
DIV, days in vitro
Dmnt, DNA methyl transferase,
DN, dominant-negative
DNA, deoxy-ribonucleic acid
DPE, days post electroporation
E12, embryonic day 12
EGF, epidermal growth factor
EGFR, epidermal growth facor receptor
ERK, extracellular signal regulated kinase
F-actin, filamentous-actin
FGF, fibroblast growth factor
FGFR, fibroblast growth factor receptor
FRS2, Fibroblast growth factor receptor substrate 2
G0, post-mitotic phase
G1, Gap 1
G2, Gap 2
Gab1, Grb2-associated binding protein 1
GABA, γ-aminobutyric acid
GAP, GTPase activating protein
GDP, guanosine diphosphate
GEF, guanosine exchange factor
GFAP, glial fibrillary acidic protein
GluR, glutamate receptor
GMC, ganglionic mother cell
Gp130, glycoprotein 130
GPCR, g-protein coupled receptor
Grb2, growth factor receptor-bound protein 2.
GSK-3, glycogen synthase-3
GTP, guanosine triphosphate
HAC, histone acetylase
HDAC, histone deacetylase
HEK 293, human embryonic kidney 293
hGFAP, human glial fibrillary acidic protein
HuD, Hu antigen D
Id, inhibitor of differentiation
IGF, insulin growth factor
IL-6, interleukin 6
INM, interkinetic nuclear migration
IPC, intermediate precursor cell (also known as basal precursor)
IRS, insulin receptor substrate
JAK, janus activated kinase
LEOPARD, Lengitines, Electrocardiographic conduction abnormalities, Ocular hypertelorism, Pulmonary stenosis, Abnormal genitalia, Retarded growth, Deafness
Lfc, Lbc’s first cousin
LIF, leukemia inhibitory factor
LIFR, leukemia inhibitor factor receptor
LIS1, lissencephaly gene 1
LPA, lysophosphatidic acid
MAPK, mitogen-activated protein kinase
MEK, mitogen activated Erk kinase
Mib, mind-bomb
mRNA, messenger RNA
Na+, sodium
NB, neuroblast
NCFC, neuro-cardio-facial-cutaneous
Nde1, Nuclear Distribution factor E homolog 1
NESC, neuroepithelial stem cell
NF, neurofibromatosis
NF1, neurofibromatosis protein 1
NFI, nuclear factor I
NGF, nerve growth factor
Ngn, neurogenin
NICD, notch intracellular domain
NMDA, N-methyl-D-aspartic acid
Nrg, neuregulin
NS, Noonan Syndrome
NT-3, neurotrophin-3
N-terminal, amino-terminal
OsM, oncostatin M
PC12, rat pheochromocytoma cell line
PDGF, platelet-derived growth factor
PDGFR, platelet-derived growth factor receptor
PI-3K, phosphatidylinositol-3 kinase
Pins, partner of inscuetable
PKA, protein kinase A
PKC, protein kinase C
PLC, phospho lipase c
PNS, peripheral nervous system
pRb, product of retinoblastoma gene
PS, presinilin
PTP, protein tyrosine phosphatase
REST, RE1 silencing transcription factor
RNA, ribonucleic acid
RP, radial precursor
Rsk, ribosomal S6 kinase
RTK, receptor tyrosine kinase
SH-2, src homology 2
Shc, src homology 2 domain-containing
Shh, sonic hedgehog
SHP-2, src homology domain protein tyrosine phosphatase-2
shRNA, short hairpin RNA
**Smad**, small/male tail abnormal/mothers against decapentaplegic homologs

**SOCS**, suppressor of cytokine signaling

**SOS**, son of sevenless

**S-phase**, Synthesis of DNA phase

**STAT**, signal transducer and activator of transcription

**SVZ**, subventricular zone

**Tctex-1**, T-complex testis expressed-1

**TGF**, transforming growth factor

**TJ**, tight junction

**Trk receptor**, tyrosine kinase receptor

**VZ**, ventricular zone
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Molecular mechanisms regulating fate determination of cerebral cortex precursors

Chapter 1: Literature Review

A. Cerebral cortex development

Overview of thesis objectives

The adult mammalian cerebral cortex develops from a relatively small pool of stem and precursor cells, yet is composed of heterogeneous populations of neurons, astrocytes and oligodendrocytes which form complex connection networks throughout the CNS. Each of these cell types arises in a temporal order from multipotent and more restricted precursor cells which are found in proliferative zones along the cortical ventricles of the developing telecephalon (Figure 1.1). Neurons are generated first, from midgestation to birth through regulated numbers of precursor divisions, while astrocytes and oligodendrocytes are produced perinatally and postnatally, respectively. Such a feat implies that these precursors and their environments are changing over time to regulate the generation of all these cell types in precise numbers. The molecular mechanisms that regulate such events as precursor fate determination and the neurogenic to gliogenic switch is the topic of this thesis.

Briefly, neurons are the main “signaling” cells in the CNS, receiving neurotransmitter inputs and propagating action potentials to other neurons via synapses. Briefly various types of glutamatergic neurons arise mainly from the dorsal cortex and are negatively regulated by ventrally-derived interneurons (Anderson et al., 2002). The cell bodies of these different types of neurons migrate from dorsal and ventral ventricular zones to form laminar structures, but form both local and subcortical connections with other CNS neurons. Astrocytes are often thought of as supporting cells for the neurons but are also important synapse components, and their numbers in the CNS are ten times those of neurons. They play many important roles such as maintaining homeostasis, reabsorbing neurotransmitters at synaptic clefts and metabolizing neurotransmitter precursors. Oligodendrocytes are the myelinating cells of the CNS, and play important roles in isolating axons, and promoting efficient action potential conductance along axons.

It is clear that many neurological disorders can arise not only from dysfunction of these cell types but also from imbalances in their numbers. These can certainly stem from
loss or degeneration throughout life, but it has recently become appreciated that some neurological disorders including autism (DiCicco-Bloom et al, 2006), Down’s syndrome (Becker et al., 1991), epilepsy (Becker et al., 2006; Guerrini & Carrozo, 2001) and even schizophrenia (Raine, 2006; Mei & Xiong, 2008) and neurological cancers (Xie & Chin, 2008) can result from neural perturbations that occur during prenatal development. Some studies, including one presented in this thesis, have provided compelling evidence that perturbations in the generation of neuronal and glial numbers during development could contribute to such disorders. An integrated knowledge of neural precursor biology and how fate decisions are regulated is therefore a prerequisite to understanding developmental neurological disorders. It can also improve our understanding towards more efficient treatment of neural cancers, where tumor-initiating cells behave like neural stem cells (reviewed in Xie & Chin, 2008). Furthermore, many scientists, clinicians and patients hope that an understanding of how neural stem cells generate all of these cell types in a controlled fashion during development will help in the manipulation of various sources of stem cells or endogenous adult neural stem cells for therapeutic purposes, for example in stroke or spinal cord injury settings. With these objectives at heart, the study of embryonic neural precursors and the molecular mechanisms by which they generate different cell types has been heavily pursued for many decades. With improving technologies and expanding knowledge, many very recent studies have provided exciting and novel mechanisms that regulate fundamental neural stem cell biology, some of which have already been put to use in exogenous stem cell models and clinical settings.

Overview of Murine Cortical Development

Although neural stem cells have been studied in many invertebrate and vertebrate organisms, the rodent cerebral cortex has been the model of choice to study fate decisions in neural stem cells for many different reasons. First, the temporal genesis of neurons, astrocytes and oligodendrocytes is well characterized. Neurons are generated from embryonic day 12 (E12) to E18 (Gillies & Price, 1993), astrocytes appear just before birth, and oligodendrocytes are first seen postnatally. Second, this temporal pattern of cell genesis can be replicated when precursors are cultured in vitro (Qian et al, 2000). Third, the fact that the cortex develops late in embryogenesis compared to other CNS structures such as the
spinal cord or brainstem, and that it is superficially localized within the embryo, make it relatively more easily amenable to in utero manipulation and dissection of precursors. Lastly, gene targeting and transgenic technologies have allowed to model many human genetic diseases in the mouse and to ask about the functional requirements for thousands of genes over the last decade. Nonetheless, invertebrate models such as drosophila and other mammalian systems such as the chick neuroepithelium or rodent spinal cord have made important contributions to our understanding of the molecular mechanisms regulating fate determination in neural stem cells and, although the work in this thesis pertains to rodent development, these will also be discussed in this chapter as appropriate.

In a gestational period of 18-20 days (depending on the strain) in mice, the cerebral cortex develops from a thin layer of ectodermally-derived neuroepithelium consisting entirely of mitotically active stem cells at E8-9. At this stage, many signaling events regulated by gradients of diffusible morphogens such as sonic hedgehog (Shh), fibroblast growth factor 8 (FGF8), WNTs, and Bone Morphogenetic Proteins (BMPs) pattern and organize the neural tube and telecephalon along a dorso-ventral as well as anterior-posterior axis. In the prospective telecephalon, these morphogens initiate the expression of key transcription factors such as Pax6 and Emx2 which specify dorsal precursors, as well as ventral-specific transcription factors such as Dlx1,2,5, Gsh2, Mash1, Lhx6 and Nkx2.1 which specify subpallial precursors. Ventral and dorsal precursors generate different neuronal and glial cell types where, for example, the majority of GABAergic interneurons are generated ventrally while glutamatergic neurons are generated from dorsal precursors. (reviewed in Lee & Jessell, 1999; Grove & Fukuchi-Shimogori, 2003; Campbell et al., 2003).

How do these relatively few stem cells then go on to generate the entire brain architecture? Many studies have provided good evidence that this occurs through a series of symmetric and asymmetric divisions which serve to amplify and generate other types of precursors (which will be discussed below), forming two zones of mitotic cells, the ventricular and subventricular zones. As daughter cells from each of these zones exit the cell cycle and begin differentiating into neurons, a third “transitional” layer called the intermediate zone forms. This is where neurons begin to differentiate morphologically and turn on neuronal genes as they migrate out to their appropriate cortical layers. The adult cortex consists of six neuronal layers, characterized by different neuronal subtypes that are
generated in an inside-out fashion, where early-born neurons are found in the inside layers while later born neurons migrate past them and fill in the outer-layers (Rakic, 1974). The first neurons that migrate into the cortex form the primordial plexiform layer. As the subsequent wave of neurons migrates out, this layer is split to form the marginal zone and the subplate. The marginal zone becomes layer I in the mature cortex, which is the outermost layer underlying the pial surface, while the subplate lies under the newly-migrating neurons. While the ventricular zone (VZ) and neuronal precursor pool expand during early neurogenesis, the VZ is eventually depleted, and is essentially lost around birth. Cortical precursors therefore undergo a defined number of cell cycles during neurogenesis (Takahashi et al. 1995) and the laminar identity of neurons is closely linked to the cell cycle number in which they were born. For example, neurons destined for layer VI and V are born in cycles 1-8, and those destined for the upper layers IV/III/II are born in the last cycles, 9-11 (Takahashi et al. 1996, 1999). (Illustrated in Figure 1.1)

Although at least some of the early neural precursors are multipotent, capable of self-renewal and generation of neuronal and glial cell types (Temple and Davis, 1994; Reid et al., 1995; Qian et al., 1998), it appears that populations of precursors become progressively restricted in their fate potential as cortical development proceeds (Qian et al., 2000; Desai & McConnell, 2000; Morrow et al., 2001; Shen et al., 2006). How these fate restrictions are imposed on cortical precursors and their progeny has been a major focus in the field, and it has now become clear that a heterogeneous population of precursors generates the cellular diversity of the adult brain.

A heterogeneous population of precursors contribute to neural diversity

Throughout cortical development, precursors alter their modes of division to regulate the fate of their daughter cells, and to balance self-renewal versus differentiation. Molecular mechanisms have evolved to control neural precursor expansion prior to neurogenesis, and to allow for an exponential generation of neurons while ensuring maintenance of sufficient precursors to produce later-born neurons, glial cells and adult neural stem cells (reviewed in Götz & Huttner, 2005; Zhong & Chia 2008). A heterogeneous population of precursors, dividing either symmetrically to generate cells of similar fates or asymmetrically to generate
cells of different fates (Figure 1.2), plays roles at each of these steps, and has only recently started to be defined.

a) Neuroepithelial stem cells

The neuroepithelial stem cells (NESCs) directly or indirectly generate all of the neurons and astrocytes in the cortex, as well as a significant proportion of the oligodendrocytes. NESCs are derived from neurectoderm, and form a thin pseudostratified proliferative zone termed the ventricular zone, which at E8-E9 represents the entire neuroepithelium. They are bipolar cells, with an apical and basal process contacting both the ventricular (apical) and basal (pial) surface respectively. The position of their soma within the ventricular zone corresponds to their cell cycle status. In a process called interkinetic nuclear migration (Sauer, 1935), the NESC soma moves from the apical (ventricular) surface where it undergoes mitosis towards the basal side (pial surface at this stage) where it undergoes S phase. This zone therefore appears pseudostratified as precursors are not synchronized in the timing of their cell cycle (Takahashi et al., 1993; reviewed in Caviness et al., 2003). Clonal analyses performed by Qian et al. showed that when these precursors are isolated at early stages of development (E10), they generate both neurons and astrocytes, and oligodendrocytes in a temporal pattern similar to that observed in vivo (Qian et al., 2000). NESCs are therefore considered multipotent stem cells. Recent work has shown that, although NESCs can generate neurons directly, the majority of cortical neurons and astrocytes are derived through an intermediate type of precursors, the radial precursors (reviewed in Gotz & Pinto, 2007). NESCs therefore undergo rapid symmetric divisions to self-renew and amplify the precursor pool in early stages of cortical development, but can also divide asymmetrically to generate other types of precursors and neurons.

b) Radial Precursors

By as yet undefined mechanisms, multipotent neuroepithelial stem cells in the nascent ventricular zone “transition” to radial precursors (RPs), which are observed as early as E9-10 and become the predominant ventricular zone precursor population during neurogenesis. RPs share many characteristics of neuroepithelial cells including the expression of antigenic proteins such as nestin & Ki67 and an apico-basal polarity that is required for their
maintenance as precursors (reviewed in Gotz & Huttner, 2005). They both share an epithelial morphology, and have adherens junctions (AJ) and tight junctions (TJ) which provide important adhesive contacts between neighboring precursors (Chenn et al., 1998), as well as intracellular links to actin cytoskeletal regulation and signaling pathways regulating gene transcription (reviewed in Hartsock & Nelson, 2008), and which, as discussed in a later section, are required for precursor maintenance. Both types of ventricular zone precursors undergo interkinetic nuclear migration and divide at the apical ventricular surface as described above, but radial precursor somas are restricted to the VZ during interkinetic nuclear migration whereas NESC somas can migrate more basally (Takahashi et al., 2002). Although unique NESC antigenic markers have yet to be identified, RPs can be distinguished from NESCs by a few features.

Radial cells were first described over a hundred years ago by pioneering neuroanatomists as a morphologically distinct subset of ventricular zone cells with long radial processes that reached the pial surface of the cortex. They were observed throughout the CNS at the onset of neurogenesis, but were first thought to solely serve as guides for radial neuronal migration (Rakic, 1972; Levitt & Rakic, 1980) and to transform into astrocytes when neurogenesis and neuronal migration were complete (Voigt, 1989; Culican, 1990). Radial precursors are now defined by their characteristic morphology, consisting of an endfoot maintaining contacts at the ventricular surface, an oval-shaped nucleus found in the VZ or SVZ, and a long radial fiber that extends just below the pial surface throughout cortical development (reviewed in Rakic, 1995; Bentivoglio and Mazzarello, 1999). Intensive monitoring of these radial fibers during cell divisions has shown that this radial fiber does not retract or divide, but is rather entirely inherited by one daughter cell. Although this issue remains contentious, this fiber is typically inherited by the neuronal daughter cell during asymmetric divisions (Miyata et al., 2001), providing a mechanism by which newly-born neurons develop a pial connection which assists in their nuclear/soma translocation as they migrate. The other radial precursor daughter cells must therefore regrow its radial fibers after mitosis, which may explain why some radial precursors have shorter radial fibers. On the other hand, these could also represent a subtype of radial precursors (Gal et al., 2006), but this remains unclear.
Unlike NESC's, radial precursors also express reactive astrocyte-specific genes such as *glast* (Shibata et al., 1997), *blbp* (Feng et al., 1994; Kurtz et al. 1994) and *gfap* in primates (Levitt & Rakic, 1980) but not rodents (sancho-Tello et al., 1995), and were therefore first termed radial glia and considered to be glial progenitors. In addition to these histological markers, RPs also express RC2 (Misson et al, 1988a), phospho-vimentin (4A4, Kamei et al., 1998), and Pax6 (Gotz et al., 1998; Noctor et al., 2002), which are not expressed in astrocytes.

Although BrdU labeling and retroviral experiments had shown that radial glial cells actively divided throughout cortical development, including during the period of neurogenesis (Misson et al., 1988b; Gray & Sanes, 1992; Halliday & Cepko, 1992; Gaiano et al., 2000), it wasn’t until the turn of the millennium that lineage tracing studies proved that RPs could generate both neurons and astrocytes sequentially (Malatesta et al., 2000; Hartfuss et al., 2001; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001), and that most ventricular zone precursors are indeed radial precursors (Noctor et al., 2002). Thus, radial precursors generate the majority of neurons and also serve as migrating scaffolds for postmitotic neuronal progeny. When neurogenesis is complete, radial precursors generate astrocytes. Many recent studies have converged to reveal a complex interplay of molecular mechanisms that govern the neurogenic to gliogenic switch in radial precursors, which is the focus of the first part of this thesis project.

It is now clear that radial precursors themselves represent a heterogeneous population of precursors, with distinct fate potentials (Hartfuss et al., 2001; Li et al., 2004). While it has been difficult to prospectively identify each subtype using antigenic markers, recent studies using various precursor-specific marker promoter-driven reporters such as sox- (Barraud et al., 2005), *nestin-* (Mignone et al., 2004), *hgfap-* (Platel et al., 2008) or *blbp* (Schmid et al., 2006) promoter driven reporters and fluorescence-activated cell sorting (FACS), have allowed for the isolation or live imaging of precursors with differing potential (reviewed in Pinto & Gotz, 2007). Although some are multipotent or at least bi-potent, clonal analysis has revealed that the majority of precursors generate either just neurons or astrocytes in culture and in vivo, suggesting that fate restrictions are acquired early during cortical development. Thus, a population of radial precursors is neurogenic, while another is non-neurogenic, and potentially generates astrocytes and adult stem cells. When and how such fate restrictions are imposed has been a heavily studied question.
RPs divide symmetrically to self-renew prior to neurogenesis, or divide asymmetrically during neurogenesis to self-renew and at the same time generate either a neuron or another type of precursor, the intermediate/basal precursors, which will be discussed below (Miyata et al., 2004; Noctor et al., 2004; Haubensak et al., 2004; Gal et al., 2006). At the end of neurogenesis, some radial precursors also undergo a terminal symmetric division to yield two neurons (Haydar et al., 2003). While most remaining radial precursors transform into astrocytes once neurogenesis is complete, some persist in the adult forebrain SVZ and contribute to the neurogenic adult neural stem cell niche (Bonfanti & Peretto, 2007). The signals that regulate the switch between symmetric and asymmetric modes of divisions are still poorly understood and represent an exciting area of research in this field. The nature of the astrocytic transformation by radial precursors, as well as the different populations of astrocytes remain unclear and are beyond the scope of this thesis.

c) Basal or Intermediate Precursors

Intermediate precursor cells (IPCs) originate from the asymmetric division of NESCs and radial precursors during neurogenesis (Haubensak et al, 2004; Miyata et al., 2001, 2004; Noctor et al., 2004). Unlike radial precursors, basal precursors lack apical and basal contacts, and divide in the subventricular zone (SVZ) or basal part of the ventricular zone. They generally do not self-renew, and divide symmetrically to generate two neurons, but do not persist post-neurogenesis (Miyata et al., 2004; Noctor et al., 2004; Haubensak et al., 2004; Gal et al., 2006; reviewed in Noctor et al., 2007). They contribute to expanding neuronal subpopulations from all cortical layers but are evolutionarily important for the generation of upper cortical layer neurons (Wu et al., 2005). The molecular mechanisms regulating their formation and differentiation are largely unknown. In distinction from other precursors, they transiently express the t-box transcription factor Tbr2 (Englund et al., 2005), which is required for the radial precursor to intermediate basal precursor transition (Arnold et al., 2008). IPCs also express the non-coding RNA Svet1 (Tarabykin et al., 2001), transcription factors cut-like Cux1 and Cux2 (Nieto et al., 2004; Zimmer et al. 2004; Cebulos et al., 2007), the vesicular glutamate transporter2 (VGlut2; Schuurmans et al., 2004), the transcription factor Satb2 (Britanova et al., 2005), and at some stages, higher levels of the neurogenic bHLH transcription factor Ngn2 (Miyata et al., 2004). Expression of some of these markers
also persists in IPC-derived upper cortical neurons (Molyneaux et al., 2007). Intermediate precursors do not express the radial precursor markers Pax6 and Hes transcription factors (Englund et al., 2005; Cappello et al., 2006), which as discussed further below, could explain the lack of self-renewal potential in this precursor population.

d) Oligodendrocyte precursor cells (OPCs)

While oligodendrogenesis is not the focus of this thesis, it is important to note that the oligodendrocytes which populate the adult telecephalon originate from different populations of precursors. First, evidence from cre/loxP fate mapping studies suggest that dorsally-derived radial precursors, in addition to generating neurons and astrocytes, can also generate oligodendrocytes (Malatesta et al., 2003). Another population of oligodendrocyte precursor cells is generated in the ventral telecephalon, and migrates dorsally during cortical development (He et al., 2001; Rakic et al., 2003).

What regulates the generation, maintenance and differentiation of each of these precursor populations during cortical development? How are fate restrictions reversibly or irreversibly controlled? What are the important signals that ensure self-renewal and differentiation of stem cells? How is the onset of neurogenesis determined and its end coordinated with the beginning of gliogenesis? What are the molecular mechanisms regulating the cell cycle of cortical precursors and the transition from predominantly symmetrical to predominantly asymmetrical divisions in the VZ as cortical neurogenesis proceeds? These are just a few of the questions that we, and others in the field, have been investigating.

B. Molecular mechanisms regulating fate determination during cortical development

Multiple environmental signals, including diffusible growth factors, inter-cellular contacts, activity-dependent signals, as well as intrinsic cellular biases influence the fate of neural precursor cells (reviewed in Levitt et al, 1995; Miller & Gauthier, 2007; Guillemot, 2007). This section is an overview of some of the various signaling molecules, receptors and transcription factors that act concertedly to regulate fate in neural precursors. It is important to note that at the time where most of these studies were conducted, individual populations of
neural precursors were not clearly defined, and so, until recently, in the majority of investigations the distinction between the heterogeneous populations of precursors is not made. For example, ventricular zone precursors are rarely defined as neuroectodermal versus radial precursors and, in many cases, ventricular zone and subventricular zone precursors are considered as a pool of heterogeneous precursors.

**Bi. Intrinsic/intracellular mechanisms regulating neural cell fate**

The expression of many intracellular players that regulate gene transcription, cell cycle progression and precursor divisions determines in part the self-renewal, neurogenic or gliogenic potential of neural precursors (illustrated in Figure 1.2). The following summary includes some of the most important regulators of neural precursor fate determination.

**a) mitotic spindle orientation during precursor divisions**

The mechanisms that regulate the switch from symmetric to asymmetric precursor cell division during neurogenesis are not clear. One proposed mechanism is the regulation of mitotic spindle and cleavage plane orientation in dividing radial precursors, which changes over the course of cortical development and correlates with the cell fate decisions of daughter cells (Chenn & McConnell, 1995; Estivill-Torrus et al., 2002; Haydar et al., 2003). Such a model suggests that polarized membrane components and fate determinants are segregated equally or unequally between daughter cells depending on the orientation of division, and that the subcellular localization of such factors is regulated during cortical development.

This idea largely stems from extensive studies conducted in *Drosophila melanogaster* neuroblasts, a system akin to the neuroepithelial divisions described above (reviewed in Chia et al., 2008). The majority of drosophila CNS neurons are derived from neuronal progenitors called neuroblasts (NBs). NBs delaminate from a neuroepithelium layer, and divide asymmetrically along an apico-basal axis to produce two daughter cells of different sizes and identity: another neuroblast which will go on dividing in the same manner, as well as a ganglion mother cell (GMC) which will divide to generate two neurons or two glial cells. The fate of dividing neuroblasts is based on a set of well-defined polarity and signaling proteins that regulate the apical vs basal localization of fate determinants and coordinates
mitotic spindle orientation to ensure preferential segregation of these fate determinants during mitosis (reviewed in Wang & Chia, 2005; Zhong & Chia, 2008).

The importance of cleavage plane orientation to symmetric vs asymmetric divisions and cell fate in the developing and adult vertebrate cortex is, however, still an open question, with data arguing both for (Feng et al., 2004; Sanada & Tsai, 2005; Shu et al., 2006; Yingling et al., 2008; Zhang et al., 2004) and against such a mechanism (Konno et al., 2008; Noctor et al., 2008; reviewed in Zhong & Chia, 2008). A few polarity and centrosomal proteins have been shown to control vertebrate precursor spindle orientation and fate during mammalian neurogenesis, including LGN (Morin et al., 2007; Konno et al., 2008), Nde1 (Feng et al., 2004), LIS1 (Tsai et al., 2005; Yingling et al., 2008; Pawlisz et al., 2008), ASPM (Fish et al., 2006), and signaling proteins such as AGS3 and G proteins (Sanada & Tsai, 2005), and doublecortin-like kinase (Shu et al., 2006) (Illustrated in Figure 1.3).

A prerequisite to such a model would be that fate determinants are segregated symmetrically in daughter cells of similar fates and asymmetrically in daughter cells of different fates or alternatively, that mechanisms segregated in similar ways regulate the expression of fate determinants in daughter cells, leading to their specific fates. Although we now know a great deal about the important molecular signals regulating precursor maintenance, neurogenesis and gliogenesis, very little is known about how or if they are differentially regulated or segregated in radial precursor progeny following mitosis. In this regard, two groups of important fate determinants that are known to be asymmetrically localized in ventricular zone precursors and may therefore be unequally segregated during asymmetric divisions, are the apically-localized Par complex proteins and adherens junctions-associated proteins, which will be discussed next.

One must keep in mind though, that not all fate determinants are polarized during mitosis, and that the notion of symmetric versus asymmetric divisions can not account for all fate decision mechanisms. It has also been proposed that, following mitosis, the length of time that a daughter cell is exposed to fate determinants and to environmental cues could also influence its fate, and this idea will be discussed in more detail at the end of this section.
b) **intrinsic factors that regulate precursor maintenance**

i) **factors that regulate cell polarity and adhesion**

As mentioned above, NESCs and radial precursors have a polarized epithelial morphology consisting of apical endfeet that are in contact with the ventricular fluid and basal fibers that are in contact with the extracellular matrix (Rakic, 2003). Additionally, apico-basal polarity is conferred by apically-localized components of the conserved Par-complex as well as cadherin-based-adherens junctions which anchor apical end-feet of neighboring VZ precursors to maintain the neuroepithelial integrity of the VZ (Chenn et al., 1998). In contrast, basal precursors which do not self-renew, do not share these characteristics (Attardo et al., 2008), suggesting that the apical junctions and intrinsic polarity of ventricular zone precursors are important for self-renewal and maintenance of neurogenic ventricular zone precursors (reviewed in Doe, 2008).

Consistent with this relatively new idea, components of evolutionarily-conserved Par-complex proteins including Par3, Par6, aPKC and Cdc42 have been found to be highly expressed in asymmetrically dividing apical precursors, localizing to the apical side of the neuroepithelium in early neurogenesis while their levels decline in late neurogenesis (Cappelo et al., 2006; Imai et al., 2006; Costa et al., 2008) (Figure 1.3). Genetic knockdown of Par complex proteins such as Par3 or aPKC in cultured or in vivo precursors leads to premature cell cycle exit and reduced proliferation (Costa et al., 2008; Imai et al., 2006), while overexpressing Par3 or Par6 leads to increased symmetric divisions in radial precursors (Costa et al., 2008) suggesting that they are required for maintaining precursors in a proliferative state. Similarly, experiments in which cdc42 was conditionally ablated prior to neurogenesis revealed an important role for this Rho-GTPase in the maintenance of apical Par3-Par6-aPKC complex as well as adherens junctions-associated proteins including cadherins, β-catenin and F-actin localization, loss of which led to failure of apically-directed INM, loss of pial attachment, basally-located divisions, acquisition of basal progenitor fates and a subsequent increase in neurogenesis (Cappello et al., 2006; Chen et al., 2006). Furthermore, cdc42 mutant embryos displayed holoprosencephaly, a common human forebrain developmental abnormality (Chen et al., 2006). These studies indicate that expression of apical polarity markers and adherens junctions is important to neural stem cell maintenance and brain development, and that both are regulated by the Rho-GTPase cdc42.
Numb and numbl are also required for the maintenance of adherens junctions and for the neuroepithelial organization of the VZ. This function appears to be independent of their roles in Notch inactivation (Rasin et al., 2007), which also regulates fate in the developing brain (discussed in later section). In the murine neuroepithelium, Numb localizes with adherens junctions in apical endfeet, as well as with trafficking vesicles associated with adherens junctions, suggesting that it may regulate trafficking of transmembrane proteins such as the cadherins to apical adherens junctions. In this regard, genetic inactivation of numb and numbl caused a loss of adherens junctions, mislocalization of cadherins, and disruption of radial morphology and polarity. As a consequence, much of the neuroepithelial organization of the ventricular zone was disrupted, leading to the dispersion of radial precursor mitoses throughout the VZ and cerebral wall (Rasin et al. 2007), suggesting that the function of Numb and Numbl in adherens junctions maintenance is important for ventricular zone integrity.

Could this have any relevance to neurological cancers? The asymmetric localization of numb is known to be important in regulating asymmetric vs symmetric divisions in the developing neuroepithelium (Shen et al., 2002). In this regard loss of lgl1, another polarity protein that interacts with Par6 and aPKC, leads to the loss of asymmetrically localized numb and perturbation in Notch signaling. Akin to the numb and numbl inactivation phenotypes, this leads to neural precursor defects in cell cycle exit, and increased number of proliferating cells that form neuroblastic rosette-like structures (Klezovitch et al., 2004). Thus, loss of polarity and asymmetric localization of fate determinants in ventricular zone precursors can lead to uncontrolled proliferation, in events similar to tumor-formation.

Consistent with these findings, many studies have shown that adherens junction-associated proteins serve as a crucial link between cell-adhesion and cell proliferation during cortical development. Adherens junctions (AJs) consist of a transmembrane cadherin protein that regulates the underlying actin cytoskeleton via interactions with β-catenin and α-catenin (reviewed in Hartsock & Nelson, 2008). This complex appears to regulate important cell-cell interactions, but probably also regulates precursor proliferation by controlling the availability of cytoplasmic β-catenin, which plays a dual role as a transcriptional factor in Wnt signaling. In this context, β-catenin associates with TCF/LEF family members to direct transcriptional activation of Wnt target genes (Clevers and van de Wetering, 1997). Disruption of N-
cadherin localization or expression in ventricular zone precursors leads to massive cortical disorganization and increased cell proliferation (Teng et al. 2005; Rasin et al., 2007), while its overexpression results in reduced precursor proliferation and increased neuronal differentiation (Noles & Chenn, 2007). Similarly, ventricular zone precursors mutant for the AJ-associated α-catenin show faster cell cycle progression, resulting in increased generation of both precursors and neurons, and overall increased brain size (Lien et al., 2006). A recent study has suggested that β-catenin signaling is restricted to VZ precursors and downregulated in SVZ precursors (Woodhead et al., 2006), raising the possibility that it regulates self-renewal. Accordingly, overexpression of β-catenin or inhibition of its degradation in precursors causes an expansion of the precursor pool and overgrowth of the cortex (Chenn & Walsh, 2002, 2003; Zechner et al. 2003), while inhibition of β-catenin causes premature cell cycle exit and neuronal differentiation (Woodhead et al. 2006). Inhibition of β-catenin signaling or overexpression of N-cadherin in VZ precursors also led to a decreased proportion of divisions yielding radial precursors, but increased the generation of Tbr2-positive, basal precursors (Noles & Chenn, 2007), suggesting that fine-tuning of cytoplasmic β-catenin levels is crucial to proper cortical development. How β-catenin regulates these effects is unclear at this point.

The above studies demonstrate that intrinsic polarity and apical contacts of NESCs and of radial precursors are essential for their maintenance as ventricular zone precursors. How these proteins are asymmetrically segregated during neurogenesis is unclear, but the asymmetric localization of the apical polarity proteins and adherens junctions-associated proteins in VZ precursors certainly provides a potential mechanism to link changes in division symmetry and fate decisions (Chenn & McConnell, 1995; Zhong et al., 1997; Shen et al., 2002; Kosodo et al, 2004; Cappello et al., 2006). Under this model, the daughter cell acquiring the apical proteins in an asymmetric division would maintain a precursor identity while the daughter cell that lacks these components could go on to differentiate. This model would also imply that the basal process would most likely be inherited by the differentiating cell, which is consistent with studies suggesting that neurons inherit radial fibers (Miyata et al., 2001). On that note, it does not appear that inheritance of basal membrane components affects cell fate (reviewed in Gotz & Pinto, 2007). However, such a model remains to be proven, and is most likely more complex.
On this note, another model for the asymmetric inheritance of fate determinants during neurogenesis has been proposed, and involves a mitotic spindle-independent process. In this process, apical membrane components of dividing precursors become asymmetrically distributed due to a regulated loss of a small prominin-rich portion of the membrane (Kosodo et al., 2004; Marzesko et al., 2005). As such, apical fate determinants are inherited asymmetrically following a vertical or perpendicular division due to a regulated loss of prominin (aka CD133)-rich portion of the membrane (reviewed in Huttner & Kosodo, 2005). The importance of such a model remains to be determined, but suggests that the regulation of symmetric and asymmetric divisions involves precisely controlled mitotic spindle and cleavage orientation and partitioning of apical fate determinants that constitute a small portion of the plasma membrane.

Little is known about what controls the expression of apical polarity and signaling proteins in ventricular zone precursors and in subventricular zone precursors. This likely is regulated by another class of fate determinants, the transcriptional regulators, which will be reviewed in the following section.

ii) transcriptional regulation of precursor maintenance

Throughout cortical development, the expression of various transcription factors controlling self-renewal and proliferation is tightly regulated (reviewed in Shi et al., 2008; Doe et al., 2008). A recurrent mechanism for regulating the timing of neurogenesis and gliogenesis is shifts in the balance of active transcription factors that promote self-renewal, and at the same time repress neurogenesis and gliogenesis, or vice versa.

Perhaps the most important family of transcription factors known to promote neural precursor identity and self-renewal is the SoxB1 proteins, including Sox1, Sox2 and Sox3 (reviewed in Wegner & Stolt, 2005). These HMG-box type of transcription factors are expressed in embryonic as well as adult neural stem cells (Zappone et al., 2000; D’amour & Gage, 2003; Graham et al., 2003; Ferri et al., 2004; Wang, T.W. et al., 2006). Reducing SoxB1 protein expression in the chick and mouse neuroepithelium leads to precocious neuronal differentiation and to the depletion of the precursor pool, while misexpression of
SoxB1 proteins blocks neuronal differentiation and maintains the precursor pool (Bylund et al., 2003; Graham et al., 2003; Ferri et al., 2004; Bani-Yaghous et al., 2008). However, increasing SoxB1 protein expression is not sufficient to maintain proliferation (Bylund et al., 2003), suggesting that these transcription factors are instead required to maintain an undifferentiated precursor state. Sox2 hypomorphic mouse mutants also show profound defects in adult neurogenesis, most likely due do a depletion of the adult neural stem cell pool during embryogenesis (Ferri et al., 2004). As in non-neural cell types (Kamachi et al., 2000; Ludwig et al., 2004; Wissmuller et al., 2006), cooperative interactions with other transcription factors are most likely required for the function of Sox proteins in neural precursors. For example, SoxB1 transcription factors cooperate with Brn-2, a POU-type homeodomain transcription factor, in regulating the neural precursor-specific nestin enhancer in the embryonic mouse spinal cord (Tanaka et al., 2004). SoxB1 transcription factors also promote precursor maintenance by antagonizing a proneural class of transcription factors, the bHLH Mash1 and Ngn5 (discussed in next section) which promote neuronal differentiation (Bertrand et al., 2002; Bylund et al., 2003; Ge et al., 2006). They likely do so by binding and sequestering the proneural proteins, inhibiting their ability to bind to DNA or to transactivate target promoters (Wegner & Stolt, 2005). On the other hand, proneural or neurogenic bHLHs can in turn suppress the activity of SoxB1 proteins in neural precursors using similar mechanisms (discussed in next section), suggesting that a balance between SoxB1 proteins and neurogenic bHLHs determines the timing of neurogenesis. How this balance is regulated during cortical development is currently unknown.

The evolutionarily conserved winged-helix (a.k.a. forkhead-box, Fox, BF-1) transcriptional repressor FoxG1 also maintains precursor cells in the developing vertebrate forebrain, and prevents them from undergoing premature neuronal differentiation. This is illustrated by the size reduction of foxg1-deficient cerebral cortices resulting from premature cell-cycle exit and neuronal differentiation, which leads to the depletion of the precursor-pool (Xuan et al., 1995; Hanashima et al., 2004; Eagleson et al., 2007). Consistently, knockdown of foxg1 using short hairpin RNA alters the timing of neurogenesis (Shen, et al, 2006).

Foxg1 most likely inhibits precursor differentiation by repressing target genes which include cell cycle inhibitors such as p21Cip (Seoane et al., 2004). A recent study links regulation of Foxg1 activation with a growth factor-mediated Akt-dependent phosphorylation
of Threonine 226 site, which mediates nuclear export and inactivation of Foxg1 (Regad et al., 2007), an example where intrinsic and extrinsic modes of regulation are integrated, as will be discussed below, to inhibit or promote neuronal differentiation.

Another important class of transcription factors that regulate precursor self-renewal and maintenance is the Notch effector Hes gene family. They are homologs of Drosophila hairy and Enhancer of split (E(spl)), and are part of the repressor-type family of bHLHs whose expression can also be regulated by Notch (reviewed in Kageyama et al., 2007) (Notch signaling will be discussed in a subsequent section). These proteins are made up of several domains: the conserved bHLH domain in the N-terminus, a proline-rich basic region, the orange domain, and the c-terminal WRPW domain which mediate the dimerization, high affinity DNA binding to the N box CACNAG sequence, protein-protein interactions, and the repressor functions respectively (Sasai et al., 1992; Dawson et al., 1995; Grbavec & Stifani, 1996). The repressor domain (WRPW domain) interacts with the corepressor TLE/Grg, a homolog of Drosophila groucho, which recruits a histone deacetylase (HDAC) to cause local chromatin condensation and inactivation of gene transcription (reviewed in Chen & Courey, 2000).

Of the seven Hes family members, Hes1, Hes3 and Hes5 are highly expressed in rodent neural stem cells (Sasai et al., 1992; Akazawa et al., 1992; Allen et al., 1999). Loss- and gain-of-function experiments in rodent precursors have provided evidence that these proteins are required for the maintenance of ventricular zone precursors. First, genetic ablation of both Hes1 and Hes5 in the embryonic brain results in the depletion of some radial precursors, and in the premature differentiation of neurons (Ohtsuka et al., 1999; Ishibashi et al., 1995; Cau et al., 2000; Hatekeyama et al., 2004). When Hes3 is also inactivated, neuroepithelial cells prematurely differentiate into neurons as early as E8.5, and by E10 the majority of radial precursors have differentiated into neurons at the expense of the later born neurons, astrocytes and oligodendrocytes (Hatekeyama et al., 2004), suggesting that Hes genes function redundantly during development, and are required to keep neural stem cells from differentiating into neurons before sufficient precursors have been generated to produce appropriate numbers of all cell types. Conversely, misexpression of Hes1, Hes3 or Hes5 in neural precursors inhibits neuronal differentiation and maintains radial precursors (Ishibashi et al., 1994; Hirata et al., 2000; Ohtsuka et al., 2001). Although their activity and
expression are regulated by Notch, their expression in ventricular zone precursors is critical to the self-renewal competence of these cells. Interestingly, intermediate precursors do not express high levels of Hes5 (Basak & Taylor, 2007), suggesting that indicators of high notch activity such as Hes1 or Hes5 expression may identify populations of precursors, such as the radial precursors, with self-renewal potential while it is absent in non-self-renewing precursors. It is therefore possible that lack of repressor-bHLH expression in Tbr2+ve basal precursors results in their non-self renewal and symmetric division into neuronal progeny (Basak et al., 2007). A group of Hes-related bHLH genes Hesr1/Hey1 and Hesr2/Hey2 are expressed in ventricular zone precursors, and also suppress neuronal fates while promoting self-renewal during early corticogenesis or astrocytic fates during mid-corticogenesis (Sakamoto et al., 2003).

How do Hes bHLHs maintain neural stem cells and regulate the timing of differentiation? They likely do so by either repressing the premature onset of the activator-type proneural bHLHs genes, which we have already started to discuss, or by repressing their activity at neuronal promoters. Hes transcription factors can bind directly to the promoter of proneural bHLHs genes such as Mash1, where it recruits the Grg/HDAC complex to repress its transcription (Chen et al., 1999). Furthermore, they can also antagonize the proneural bHLHs by forming inhibitory heterodimers with E-proteins, thereby sequestering this bHLH co-activator and inhibiting its heterodimerization with proneural bHLHs (Sasai et al., 1992).

This function is reminiscent of another class of inhibitory bHLHs, the Id (a.k.a. inhibitor of differentiation) proteins, which lack a DNA binding domain and thereby act as dominant-negative bHLHs (reviewed in Tseng, 2003). Id1, Id2 and Id3 mRNA are highly expressed in proliferating but not differentiating precursors during early cortical development (Duncan et al., 1992; Nagata & Todokoro, 1994; Jen et al., 1997). Targeted disruption of both Id1 and Id3 in mice results in premature withdrawal of precursors from the cell cycle and expression of neural differentiation markers (Lyden et al., 1999; Bai et al., 2007), suggesting that these Id genes are required for proliferation and normally regulate the timing of neurogenesis. Similarly, overexpressing Id genes blocks neurogenesis and oligodendrocyte formation, but does not interfere with astrocyte formation (Kondo & Raff, 2000; Toma et al., 2000; Nakashima et al., 2001). Id proteins can promote proliferation and inhibit neurogenesis via multiple mechanisms. They can promote cell cycle progression by
sequestering pRb from its inhibitory interactions with E proteins, where E proteins are required for the transcription of genes linked to cell cycle progression, (Iavarone et al., 1994; Lasorella et al., 2000; Toma et al., 2000). Ids also inhibit neurogenesis by sequestering the requisite co-activator E proteins from proneurogenic bHLHs (Norton, 2000), or by sustaining the expression of other inhibitory bHLHs such as the Hes genes (Bai et al., 2007). Although Id expression appears to be upregulated by extracellular signals such as BMP-activated Smads at some developmental stages (Nakashima et al., 2001; Ying et al., 2003), the signals that promote Id degradation and suppress its expression at the onset of neurogenesis remain elusive.

Another transcription factor that likely regulates important aspects of radial precursors is Pax6, one of the antigenic markers unique to radial precursors. Not surprisingly, extensive studies have focused on uncovering the roles of this transcription factor in regulating the identity and functions of this type of precursors. (reviewed in Guillemot et al., 2006). Pax6 plays multiple roles in CNS development, as illustrated by the severe brain malformation and missing eyes of mice and humans carrying homozygous Pax6 mutations (Hogan et al., 1996; Glaser et al., 1994). Key roles include brain patterning, neuronal specification, neuronal migration, and axon projection (reviewed in Osumi, 2001; Simpson & Price, 2002; Guillemot et al., 2006)

Pax6 is a transcription factor that contains two DNA binding domains, the paired-box domain and the homeobox domain (Bopp et al., 1986; Treisman et al., 1991), and is expressed throughout cortical development, including at the initial stages of brain development preceding neurogenesis (Stoykova & Gruss, 1994; Inoue et al., 2000; Stoykova et al., 1996; Caric et al., 1997), where it regulates stem cell maintenance. At the onset of neurogenesis, Pax6 is expressed by the majority of radial precursors (Malatesta et al. 2000; Hartfuss et al., 2001), where it also plays a role in regulating neurogenesis as will be discussed in the next section. During early stages of cortical development, Pax6 is required to control cell cycle duration, the rate of progression from symmetric to asymmetric spindle-oriented divisions and the onset of neural-specific markers (Gotz et al., 1998; Warren et al., 1999; Estivill-Torrus et al., 2002). Pax6 may regulate precursor maintenance by regulating the expression of target genes such as fabp7, the gene encoding BLPB, brain lipid binding protein (Arai et al., 2005) but also promotes neurogenesis by transcriptionally regulating
Ngn2 (Scardigli et al., 2003). Misexpression of Pax6 in the early neuroepithelium mainly results in ectopic Fabp7 expression, but not Ngn2, suggesting that Pax6 functions are highly context-dependent. Furthermore, downregulation of Fabp7 results in perturbed cell proliferation and increased neuronal differentiation, suggesting that Pax6 regulates genes that are essential for the maintenance of neuroepithelial cells during early cortical development (Arai et al. 2005). Consistently, Pax6 also positively regulates Sox2 expression (Wen et al., 2008), a transcription factor that promotes stem cell maintenance, and as such is required for neurosphere formation. Pax6 may also play a role in regulating adhesion in ventricular zone precursors, as illustrated by the disruption in polarity and morphology of these cells in Pax6 mutants (Götz et al., 1998). Although this has not been shown directly, Pax6 may play a role in adherens junction maintenance by regulating the expression of cadherins (Tyas et al., 2003).

Pax6 is expressed by the majority of radial precursor cells at the onset of neurogenesis (Malatesta et al., 2000; Hartfuss et al., 2001) but is downregulated in the transition from ventricular zone to basal precursors (Englund et al., 2005), suggesting that it plays an important role in apical precursor maintenance and self-renewal during neurogenesis. Both loss- and gain-of-function studies have shown that Pax6 endows radial precursors with their ability to generate neurons (Heins et al, 2002; Haubst et al., 2004). The isolation of precursors from the early neuroepithelium of Pax6 mutant small eye mice (Sey/Sey) show decreased neuronal differentiation when compared to wildtype precursors, an effect which can be rescued by transfecting exogenous Pax6 (Gotz et al., 1998; Heins et al., 2002). Altogether, these studies suggest that Pax6 is essential for the maintenance of radial precursors and for their neurogenic potential. However, a major caviat of these studies is that the primary and secondary causes of the cortical defects observed in the absence of Pax6 are not well-characterized. In this regard, no acute loss of function studies have been performed, but are required to determine the role of Pax6 during neurogenesis and gliogenesis.

With regards to Pax6 as well as all of the transcription factors that were discussed in this section, much remains unknown about how these proteins regulate precursor maintenance at the transcriptional level. For example, what transcription factors regulate the expression of proteins that determine precursor polarity and adhesion, proteins that are absolutely required for neural precursor maintenance? What stem cell markers do they
regulate? Do they regulate each other? Do they simply antagonize neuronal gene expression? Do they regulate precursor responsiveness to extracellular cues, and how?

c) **neurogenic transcription factors**

Similarly, transcription factors also actively promote neurogenesis during cortical development. As discussed above Pax6 plays an important role in regulating precursor maintenance as well as neurogenesis, and in this context, functions in part by directly regulating neurogenic bHLH proteins.

This brings us to another important family of transcription factors, the evolutionarily conserved proneural bHLH family of transcription factors that are required for neurogenesis. Proneural bHLHs coordinate multiples aspects of the neurogenic process, including neuronal fate commitment/determination, maturation of precursors, neuronal specification and migration and have been the subject of extensive study in these fields of research (recently reviewed in Bertrand et al., 2002; Kageyama et al., 2005; Guillemot et al., 2007). Here, I will focus on the studies demonstrating a role for proneural bHLHs in the acquisition of a neuronal fate and suppression of astrocytic fate.

Three proneural bHLHs including neurogenin 1 (Ngn1), neurogenin 2 (Ngn2) and Mash1 are expressed in ventricular zone/subventricular zone precursors during early cortical development (Guillemot and Joyner, 1993; Lo et al., 1991; Sommer et al., 1996). Ngn1 and Ngn2 are expressed in the dorsal telecephalon, whereas Mash1 is predominantly expressed in the ventral telecephalon and expressed at lower levels in the dorsal telecephalon (Britz et al., 2006). Consistently, these transcription factors regulate glutamatergic versus GABAergic neuron specification respectively (Fode et al., 2000; Parras et al., 2002). Another group of bHLHs that includes NeuroD are induced by proneural bHLHs (Ma et al., 1996; Britz et al., 2006), and are not expressed in the early neuroepithelial precursors but rather in newly-born neurons, suggesting that they regulate neuronal differentiation post-commitment (reviewed in Bertrand et al., 2002).

In the chick and murine cortex, misexpression of bHLHs such as Nng2 or Mash1 induces premature cell cycle withdrawal and neuronal gene expression, and also inhibits the expression of astrocyte-specific genes (Sun et al., 2001; Castro et al., 2006; Britz et al., 2006), suggesting that bHLHs promote neurogenesis and negatively regulate gliogenesis.
Consistently, loss of function studies in mice have demonstrated a requirement for Ngn1 and Mash1 in neurogenic fate determination of precursors. While *Mash1*-deficient embryos display decreased neurogenesis in the ventral telecephalon (Casarosa et al., 1999; Horton et al., 1999), single or double Ngn1/2 knockouts show compensatory overexpression of Mash1 in CNS precursors (Fode et al., 2000), and mainly display PNS-associated defects in neurogenesis (Fode et al., 1998; Ma et al., 1998; Ma et al., 1999), as well as misspecification of neurons in the dorsal cortex (Fode et al., 2000; Schuurmans et al., 2004). Subsequent analysis of these mutants also revealed misregulation of several pan-neuronal markers in the cortex (Mattar et al., 2004), suggesting neurogenesis defects. Analysis of double Ngn2/Mash1 mutant precursors furthermore revealed severe defects in corticogenesis *in vivo*, including decreased neurogenesis and excessive and premature differentiation of astrocytes, resulting from defects in fate commitment of cortical precursors (Nieto et al., 2001). Thus proneural bHLHs are required for the neurogenic fate determination of precursors, and suppression of gliogenic fates. Interestingly, some neuronal precursors remained in these studies, suggesting that other proneural genes or neurogenic mechanisms are also involved in this process.

How do bHLHs execute these and other functions during neurogenesis? Activator-type bHLHs such as Ngn1 and Ngn2, Mash1 (*achaete-scute* homolog), Math1 (*atonal* homolog), and NeuroD in part control these processes by regulating the expression of many target genes. To do so, they form heterodimers with ubiquitously expressed bHLH co-factors, E12 or E47, and bind a six base pair DNA motif called E-box in the promoters of target genes (Massari & Murre, 2000), with some specificity (Bertrand et al., 2002; Powell et al., 2001), suggesting that they cooperate with other transcription factors. The nature of these interactions is still unclear.

An example of this is illustrated in one of the mechanisms by which proneural genes regulate corticogenesis. Ngn1 and Ngn2 induce the transcriptional activation of Notch ligand *Delta* and Serrate/Jagged, leading to lateral inhibition of neurogenic fates in neighboring precursors (reviewed in Bertrand et al., 2002), but by differing mechanisms. They each bind to different delta enhancers (Castro et al., 2006), where Mash1 acts synergistically with the POU transcription factor Brn in activating *delta1* transcription (Castro et al., 2006).
Proneural bHLHs also promote neurogenesis by upregulating the expression of transcription factors, such as Hes6, that function in a positive-feedback loop to stabilize/potentiate the expression and activity of proneural genes (Bae et al., 2000; Koyano-Nakagawa et al., 2000; Jhas et al., 2006), thereby altering the balance between proneural bHLHs and inhibitory factors that counteract their activity, in favor of neurogenesis. Proneural bHLHs also induce a cascade of neuronal-differentiation genes including NeuroD and Math2, which are required for the differentiation of different CNS neuronal populations (Fode et al., 2000; Mattar et al., 2004), thereby regulating sequential steps of cell determination and differentiation. Proneural bHLHs also promote cell cycle withdrawal, likely by activating the transcription of inhibitors of cell cycle progression such as p16, p21, p27 (Farah et al., 2000; reviewed in Bertrand et al., 2002).

In order to promote a neurogenic fate, precursor and glial fates must also be suppressed. One way in which the neurogenic bHLHs can promote neurogenesis is by inhibiting the activity of SoxB1 proteins, which are required for precursor maintenance. As mentioned previously, direct interactions between bHLHs and SoxB1 proteins could reciprocally sequester and inhibit their respective activities in neural precursors (Wegner & Stolt, 2005). Evidence in chick suggests that bHLHs also promote neurogenesis by upregulating the expression of another neurogenic transcription factor, Sox21, a member of the SoxB2 family of Sox proteins, which counteracts the activity of Sox1, 2 and 3 (Sandberg et al. 2005). SoxB2 proteins, which also includes Sox14, are closely related to the SoxB1 proteins discussed in the previous section, and most likely bind similar target sequences, but possess a repression domain instead of a C-terminal transactivation domain (Uchikawa et al., 1999), suggesting that they may turn off the genes that SoxB1 proteins transactivate. In accordance with this idea, Sox21 is expressed at variable levels in chick ventricular zone precursors that are Sox1-3-positive, but is downregulated in differentiating neurons (Uchikawa et al., 1999; Sandberg et al., 2005). Misexpression of Sox21 in the chick neuroepithelium causes cells to exit the cell cycle, downregulate precursor markers Pax6 and Sox3, and to turn on some neuronal genes, while reducing Sox21 expression in turn inhibits neurogenesis (Sandberg et al., 2005). This effect on neuronal differentiation is dependent on the Sox21 repressor domain (Sandberg et al., 2005). Thus, the intrinsic balance between SoxB1 and SoxB2 protein expression and activity likely determines whether a precursor...
remains undifferentiated or commits to neuronal differentiation, and this balance is at least partially regulated by increasing levels of proneural bHLHs at the onset of neurogenesis.

The mechanisms by which proneural bHLHs suppress the astrocytic fate however most likely differ from its transcriptional regulatory roles in neurogenesis. As will be discussed in the next section, gliogenic cytokine-induced astrocyte formation involves the formation of a complex between phosphorylated STAT transcription factors, Smads and co-activator p300/CBP (Nakashima et al., 1999a). Ngn1 blocks astrocyte formation by competing and interfering with the formation of this complex, where an Ngn/Smad/p300/CBP complex formation promotes neurogenesis during early corticogenesis. Ngn1 can also inhibit phosphorylation of the STATs by unidentified mechanisms (Sun et al., 2001).

While they are required for neuronal commitment, new functions have recently been identified for these bHLHs in later cortical development. Consistently, the expression patterns of proneural bHLHs are altered as corticogenesis proceeds. In the dorsal cortex at E12.5, a few VZ precursors have started to express the bHLHs, and the majority of these co-express ngns and Mash1. At this time point, proneural bHLHs are thought to play a role in fate commitment and restriction of fates in precursors (Britz et al., 2006; Nieto et al., 2001). An interesting expression pattern becomes strikingly noticeable in the ventricular zone during mid-corticogenesis. At E15.5, precursors at the ventricular surface or at the VZ/SVZ boundary, express Mash1, whereas Ngn1/2+ve cells are restricted to the central part of the VZ (Britz et al., 2006). This suggests that proneural bHLH expression is dynamically regulated throughout the cell cycle, as precursors undergo interkinetic nuclear migration (INM), and that the transition from radial to intermediate precursors could be regulated by this family of transcription factors. Accordingly, Mash1 and Ngn2 were found to be relatively more highly expressed in early and late G1/G0 respectively (Miyata et al., 2004; Britz et al., 2006). Analysis of Ngn2 knockouts at this but not earlier time points revealed an increase in mitotic precursors in the SVZ and concomitant decrease in the VZ (Britz et al., 2006), suggesting that Ngn2 regulates the transition of radial precursors to intermediate precursors during mid-corticogenesis. In these mutants, Mash1 expression is also upregulated (Fode et al., 2000; Schuurmans et al., 2004), and consistently, overexpression of Mash1 in the dorsal VZ at E14.5 promotes a VZ to SVZ precursor transition, without altering
neuronal specification at this time point (Britz et al., 2006). Thus, at mid-cortical stages, Ngns and Mash1 cooperate to regulate cortical precursor transitions, a function that is distinct from their neurogenic fate and neuronal specification functions during early corticogenesis. How these different functions are regulated is currently unknown, and is evidently highly dependent on cellular context.

The C/EBPs (CCAAT/Enhancer-binding protein) are part of another family of transcription factors (reviewed in Ramji & Foka, 2002) that promote neurogenesis, and at the same time repress gliogenesis (Ménard et al., 2002; Paquin et al., 2005; reviewed in Miller & Gauthier, 2007). C/EBPs are basic region-leucine zipper (bZIP) types of transcription factors, well known for their role in the regulation of myogenesis and adipogenesis (reviewed in Ramji & Foka, 2002; Musri et al., 2007).

C/EBP isoforms α, β and δ transcripts are expressed in the developing telecephalon at the onset of neurogenesis (Ménard et al., 2002). In this system as well as in nonneural cells, they are activated by extracellular signals that activate signaling cascades such as the MEK-ERK-ribosomal S6 kinase (Rsk) pathway (discussed in a later section), providing one link between intrinsic and extrinsic factors regulating cortical development (reviewed in Miller & Gauthier, 2007; Nerlov et al., 2008).

Our lab has proposed that subsequent to neuronal commitment by bHLHs as discussed above, extracellular growth-factor signaling-activated C/EBPs transcriptionally activate neuronal genes and promote neuronal differentiation. Overexpression of an acidic dominant-negative form of C/EBP (A-C/EBP), where the basic DNA binding domain is mutated to acidic residues, leads to the formation of C/EBP dimers that cannot bind DNA. Overexpression of this A-C/EBP in cortical precursors inhibits all C/EBPs, and results in delayed neurogenesis and increased astrocyte formation in vivo and in cultures treated with PDGF (Paquin et al., 2005; Ménard et al., 2002), suggesting that C/EBPs are required for growth-factor mediated neurogenesis. Conversely, while overexpression of a wildcard form of C/EBP did not affect neurogenesis (gliogenesis was not addressed) (Ménard et al., 2002), a known Rsk phosphorylation site phosphorylation mimick (T217E) form of C/EBP (CA-C/EBP) was sufficient to increase neurogenesis and suppress gliogenesis in cultured precursors and in vivo (Ménard et al., 2002; Paquin et al., 2005), suggesting that growth-factor-mediated MEK-ERK-Rsk signaling promotes neurogenesis and inhibits gliogenesis at
least in part by activating the C/EBP transcription factors, which are not limiting during early
cortical development. Consistently, overexpression of C/EBPs where ERK and Rsk threonine
phosphorylation sites were converted to alanine, prevented neurogenesis in culture and in
vivo, but did not affect gliogenesis (Paquin et al., 2005).

How do C/EBPs promote neurogenesis? In nonneural cell types, C/EBPs regulate
proliferation and differentiation by directly transactivating and/or repressing gene expression,
a function that is highly dependent on their posttranslational modifications and their
interactions with other factors (reviewed in Ramji et al, 2007; Musri et al., 2007; Nerlov et al.,
2008), something that has only started to be addressed in the developing nervous system.
C/EBPs form many protein-protein interactions, including dimerization with other C/EBPs
via their c-terminal bZIP domain, and interactions with the transcriptional apparatus and
transcriptional coactivators CBP/p300 via their N-terminus. C/EBPs can also interact with
Histone Deacetylases (HDACs), and thus repress transcription of target genes, as will be
discussed in the next section. C/EBPs also contain domains interacting with the SWI/SNF
nucleosome remodeling complexes, and several sites of post-translational modification. For
example, C/EBPs can be phosphorylated at many serine/threonine sites in response to
extracellular activation of p38 MAPK, GSK3, Rsk and ERK, and can also be sumoylated or
acetylated at lysine sites in its regulatory domain motif (RDM). These post-translational
modifications regulate protein-protein interactions and the transcriptional activator/repressor
functions of C/EBP (reviewed in Nerlov et al., 2008). For example, in nonneural cells,
different extracellular stimuli modulate direct interactions between C/EBPs and many other
transcriptional regulators including forkhead transcription factors (Sekine et al, 2007), Runx
transcription factors (reviewed in Nerlov et al., 2007) and various Smads (reviewed in Nerlov
et al., 2008).

Phosphorylation of the C/EBP α, β, and δ by ERK and Rsk activates them to directly
transactivate early neuronal genes such as *tα1 α-tubulin* (Miller et al., 1987; Gloster et al.,
1994; Ménard et al., 2002) as well as the neurogenic bHLH *neurol6*
(Uittenbogaard et al., 2007). The functional interactions of C/EBPs in this context, for
example in recruiting co-activators or synergizing with other transcription factors, are not
known, as are target genes other than *tα1α-tubulin* and *neurod6*. 
Outside of the nervous system, C/EBPs can also induce differentiation by promoting cell cycle exit (reviewed in Nerlov et al., 2007). Thus, C/EBPs may also regulate neurogenesis by interacting with cell cycle regulators such as cyclins, cyclin-dependent kinases, pRb as well as E2F proteins (Chen et al., 1996; Iacova et al., 2003; Sebastian et al., 2005; reviewed in Nerlov et al., 2007). In different contexts, C/EBPs can also promote or inhibit cell cycle exit by binding and regulating transactivation at the e2f or Id1 promoters (reviewed in Nerlov et al., 2007).

How do C/EBPs suppress astrocyte formation? Post-translational modifications likely regulate this function, as a non-phosphorylatable forms of C/EBP at the ERK and Rsk threonine sites could inhibit neurogenesis without affecting gliogenesis (Paquin et al., 2005), suggesting that extracellular signals likely regulate the interactions of C/EBPs with other transcription factors at specific promoters to regulate astrocyte formation. For example, in a similar model to bHLH-mediated suppression of astrocyte formation, C/EBPs may compete with gliogenic transcription factors for Smads and p300/CBP co-factors, thereby inhibiting gliogenesis; and this may require posttranslational modifications of C/EBPs. However, while there is currently no evidence that C/EBPs can bind gliogenic promoters, the experiments involving the A-C/EBP suggest that the DNA binding function of C/EBPs is also required for the negative regulation of gliogenesis.

d) gliogenic transcription factors

Evidence that a transcriptional code regulates stem cell maintenance and neurogenesis has prompted many to question whether a transcriptional code regulating the onset of gliogenesis also exists. This is still up for debate.

Until recently, the signal transducer and activator of transcription (STAT) proteins were the only family of transcription factors known to directly regulate astrocyte formation. These transcription factors have src-homology-2 (SH-2) domains that mediate their homo- or hetero-dimerization when phosphorylated at Tyr705 sites, as well as transactivation domains that interact with the transcriptional machinery. They act downstream of cytokine signaling pathway and are activated by Janus kinases (JAKs), a pathway that instructively drives astrocytes formation in response to cytokine signaling as will be discussed later. In this context, Janus kinases (JAKs) phosphorylate STATs, leading to their homo or hetero-
dimerization and translocation to the nucleus. The STATs can also be phosphorylated at serine and threonine sites in response to activated receptor tyrosine kinases, leading to modulated STAT activity (reviewed in Cattaneo et al. 1999).

STAT3 has been implicated in gliogenesis (Bonni et al, 1997; Rajan & McKay, 1998). Immunohistochemistry has revealed that STAT3 is expressed in some ventricular zone precursors as early as E10.5 (Yan et al., 2004), and is enriched in cultured E11-12 cortical precursors relative to newly born neurons (Barnabe-Heider et al., 2005), suggesting that STATs are present during the period of neurogenesis. Components of JAK-STAT signaling, including STAT3 expression as well as STAT1 and STAT3 activation increases with time in cultured precursors, suggesting that a positive autoregulatory loop upregulates the JAK-STAT pathway as development proceeds (He et al., 2005).

A cytokine-gp130/LIFRβ-JAK-STAT pathway has been implicated in the regulation of astrogenesis in cultured cortical precursors (Bonni et al., 1997; Rajan & McKay, 1998) as will be discussed in the next section. Transfection of dominant-negative forms of STAT3 blocked CNTF-mediated astrocyte differentiation and GFAP expression in E14 cultured precursors (Bonni et al., 1997; Rajan & McKay, 1998). Similarly, knockdown of STAT3 levels using siRNA decreased CNTF or conditioned media-induced astrocyte formation but increased neurogenesis in the same conditions (Barnabe-Heider et al., 2005), suggesting that STAT3 activation is required for astrocyte formation and for the suppression of neurogenesis.

How do STATs induce gliogenesis? Phosphorylated STATs can form a synergistic interaction with the BMP-activated Smads and co-activator p300/CBP and transactivate glial promoters (Nakashima et al., 1999a). Furthermore studies have shown that STATs are required for the direct transactivation of at least two astrocytic genes, *gfap* and *s100b*, via STAT binding sites within the promoters of these genes (Kahn et al., 1997; Nakashima et al., 1999a; Namihira et al., 2004). Activated STATs can only transactivate these genes during late cortical development when these promoters are transcriptionally accessible, as will be discussed in the next section. As such, in early cortical precursors, overexpression of STAT3 is not sufficient to drive astrocyte formation (He et al., 2005).

But is STAT3 a true gliogenic transcription factor? As discussed above, early cortical precursors express detectable levels of STAT3, but the role of this transcription factor in early cortical development is unclear. Targeted disruption of *stat3* in the mouse leads to
embryonic lethality (Takeda et al., 1997), demonstrating that this transcription factor is critical at early developmental stages, but leaving the question of its role in nervous system development unanswered. A floxed stat3 mouse (Takeda et al., 1998), was only recently studied in the context of CNS precursor development, and revealed another role for STAT3. Transfection of stat3^{fl/fl} E12.5 precursors with cre recombinant plasmids in culture or in vivo resulted in decreased neurosphere size in culture and decreased proliferation in vivo, and precocious neurogenesis (Yoshimatsu et al., 2006), suggesting that STAT3 also functions to regulate precursor maintenance during development. This effect was non-cell autonomous, as non-transfected cells also ectopically expressed neuronal markers. STAT3-dependent transactivation of the notch ligand delta was identified as a mechanism for this function. Thus, although STAT3 activation undeniably instructs precursors to generate astrocytes during the period of gliogenesis, at early time points, STAT3 and the JAK-STAT pathway also play important roles in the maintenance of neural precursor cells, cooperating with FGF and Notch signaling, extracellular factors that will be discussed in the next section. Another example of cross-talk between Notch and JAK-STAT signaling is illustrated by the Notch-effector hes genes, which were discussed in the previous section. The hes genes that are required for precursor maintenance and self-renewal also promote astrocyte formation during late cortical development by facilitating JAK-STAT signaling activation (Kamakura et al., 2004), and inhibiting oligodendrocyte formation by antagonizing the olig bHLHs (Wu et al., 2003; reviewed in Kageyama et al., 2008). It is thus becoming apparent that pathways regulating stem cell maintenance also promote astrocyte formation, not only by suppressing neurogenesis and ensuring that a pool of precursors remains for gliogenesis, but by regulating JAK-STAT activation.

Furthermore, while it is clear that JAK-STAT activation drives terminal astrocyte differentiation, whether STAT3 activation regulates the initial gliogenic switch in precursors is still unclear. Until recently, no transcription factor had been identified that could promote the initial astrogenic fate. In the spinal cord, one HMG-box transcription factor, Sox9, is required for the proper timing of gliogenesis, but it is not sufficient to promote gliogenesis when expressed on its own in vivo (Stolt et al., 2003; Wegner & Stolt, 2005; Deneen et al., 2006).
Recent studies suggest that the nuclear factor-I (NFI) family of CCAAT box-element-binding transcription factors, which includes NFI-A, -B, -C, and –X (reviewed in Gronostajski et al., 2000), are essential for the termination of neurogenesis and for the initiation of gliogenesis, at least in the chick neural tube. This family of transcription factors is developmentally regulated at the levels of expression and alternative splicing, and can both activate or repress transcription of target genes depending on promoter and cellular context (Gronostajski et al., 2000).

Early studies had hinted that these genes could regulate astrocyte formation. *Nfia*, *b* and *x*, but not *Nfic*, are highly expressed in the neocortex from E9 to birth, and transcripts as well as protein products can be specifically detected in the ventricular zones of the CNS and in the spinal cord and as early as E10.5 and subsequently in the cortical layers until at least E16.5 (Chaudhry et al. 1997, Shu et al., 2003; Driller et al., 2007), while they are dynamically expressed in many neural cells postnatally, (Chaudhry et al., 1997; Plachez et al., 2008; Campbell et al., 2008), including expression of *Nfix* in ependymal cells (Driller et al., 2007), suggesting that they are at least expressed by some neural precursors during cortical development. Interestingly, transcriptionally important NFI binding sites have been identified in the astrocytic-specific *gfap* promoter (Miura et al., 1990; Krohn et al., 1999; Cebolla & Vallejo, 2006; Wong et al., 2008), suggesting that this family of transcription factors could regulate astrocyte formation. Analysis of individual *Nfia* and *Nfib* knockout mice revealed several neurological abnormalities, including agenesis of the corpus callosum and hydrocephaly (das Neves et al., 1999; Steele-PERKins et al., 2005). In agreement with the expression data, *Nfic* mutants have no brain phenotype (Steele-PERKins et al., 2003). Although *Nfia* -/- adult mice display a dramatic reduction in *gfap* expression, this does not likely reflect abnormalities in astrocytic numbers, as the expression of another astrocyte marker, s100β, is unaffected (das Neves et al., 1999). Interestingly, development of midline glia, which regulate crossing of the commissural axons at the midline, is severely impaired in the *Nfia* -/- and *Nfib* -/-, suggesting that NFI proteins may regulate astrocyte formation or maturation (Shu et al., 2003; Steele-PERKins et al., 2005). However, precursor dynamics and cell genesis were not evaluated directly in these studies, and it is difficult to ascertain the cause of the observed phenotypes, which could be due to defect in either or both neuronal and glial differentiation, as well as neural precursor proliferation or neuronal pathfinding.
NFI binding sites have also been identified in the promoters of neuronal genes such as \textit{nf(m)} (Elder et al., 1992) as well as oligodendrocyte-specific genes such as \textit{mbp} (Inoue et al., 1990), and the radial precursor/astrocyte marker \textit{blbp} (Bisgrove et al., 2000), and more recently in 70 other CNS-specific genes (Wong et al., 2000), suggesting that NFIs regulate many aspects of cortical development. Therefore, these studies, although intriguing, have not been very informative about the role of NFI transcription factors in early neural precursors, and many more cellular aspects of brain development should be addressed in these mutants.

A recent study investigating the role of Nfia in chick neural tube precursors (Deneen et al., 2006) has however provided more substantial insights into its role in early cortical development. The authors of this study first found that, in the mouse spinal cord, Nfia and Nfib were only induced at the onset of astrogliogenesis, as had previously been suggested (Cebolla & Vallejo, 2006). Through loss and gain-of-function experiments in the chick neural tube, they ascertained that misexpression of Nfi genes resulted in precocious gliogenesis but did not repress neurogenesis. However, Nfia expression is required for the gliogenic switch, where ablation of Nfia results in delayed gliogenesis and prolonged neurogenesis. Nfia is also antagonized by the olig2, a transcription factor that, along with olig1, is required for oligodendrogenesis ((Lu et al., 2002; Zhou & Anderson, 2002), suggesting that a balance between these two factors regulates the specification of bi-potent glial precursors (Deneen et al., 2006).

While Nfia regulates the onset of gliogenesis in the spinal cord, it remains unclear whether Nfi genes play similar roles in the murine dorsal cortex, where they are expressed as early as E9, a time preceding gliogenesis. The recent characterization of the brain phenotype in \textit{Nfix-/-} mice may shed some light on this question. While these mice also display hydrocephalus and partial agenesis of the corpus callosum without any changes in \textit{gfap} expression (Driller et al., 2007) other brain defects included hippocampal distortion, increased dorso-ventral total brain size throughout adulthood, and ongoing increase in total brain weight with age, despite a lower total body weight. This suggests that there could be more neurons and/or astrocytes in the brains of \textit{Nfix-/-} mice (Campbell et al., 2008). The surprising finding was that, from birth to adulthood, the lateral ventricles of Nfx-/- mice were aberrantly filled with densely-packed cells, which turned out to be positive for Pax6 as well as for the neuronal precursor/migrating neuronal marker doublecortin (Campbell et al., 2008).
These could be aberrant neuronal precursors or ectopic neurons, suggesting that Nfix, like Nfia, is required to terminate neurogenesis at the onset of gliogenesis. This function may be separate from the role of Nfia in promoting astrocyte formation, as no perturbations in \textit{gfap} expression were detected in the \textit{Nfix/-} brain, at least in an independent study (Driller et al., 2007). Alternatively, Nfix may regulate neural stem cell proliferation and differentiation during embryonic development as well as in adulthood.

In a different type of study, genes that are dysregulated at the mRNA level in the early postnatal brain of Nfia-/- mice were identified by microarray analysis. Although it is not possible to summarize all of the interesting findings here, one important pattern that was observed was the respective upregulation of oligodendrocyte precursor markers and downregulation of mature oligodendrocyte markers in Nfia-/- brains, suggesting that these brains were developmentally delayed (Wong et al., 2007). Perhaps, as in the Deneen & Campbell studies where the timing of neurogenesis and gliogenesis was perturbed, oligodendrogenesis is delayed in the \textit{Nfi} mutant animals.

The role of the \textit{Nfi} family of transcription factors is only started to be dissected, and it is most likely, given its expression pattern, that new roles in neural precursor maintenance and differentiation will be uncovered. For example, it is still unknown if these proteins play redundant functions during development, as analysis of double as well as triple-knock out mice has not been done. Furthermore, quantitative analysis of cell genesis during early cortical development needs to be performed in order to unveil the true roles of \textit{Nfi} genes in neural cell genesis. Like many other transcription factors, the specificity of Nfi function is likely regulated by many protein-protein interactions and post-translational modifications. For example, NFIs may collaborate with JAK-STAT signaling during astrocyte formation, potentially by directly interacting with the gliogenic STATs, as seen in nonneural cells (Mukhopadhyay et al., 2001).

e) \textbf{Epigenetic regulation of neural precursors}

While it is clear that a battery of transcription factors act concertedly to regulate neural precursors and their fates, little is known about the mechanisms that regulate the balance between non-neurogenic vs neurogenic, or neurogenic vs gliogenic transcription factors and their activity at specific stages. Important intrinsic changes must occur during
development to adjust the neurogenic-gliogenic factor balance in precursors, and regulate their competence to generate neurons versus astrocytes. In this regard, epigenetic mechanisms play a crucial role in the regulation of gene expression and fate.

What stops a gliogenic program that is present to some degree in early neural precursors from turning on before the appropriate timing of gliogenesis? Or in the case of the *stats* and *hes* genes, how does a pathway which regulates both precursor maintenance and astrocytogenesis regulate its functions during development? How do gliogenic programs turn on and neurogenic programs turn off at the end of the neurogenic period? While we have already discussed the importance of the neurogenic bHLHs in repressing gliogenic factors, a second mechanism involves the epigenetic regulation of gliogenic genes. Epigenetic mechanisms are a relatively newly appreciated way in which gene expression and timing of fate decisions are regulated in neural stem cells (reviewed in Feng et al., 2007; Namihira et al., 2008) “Epigenetic mechanisms refer to any heritable influence (in the progeny of cells or individuals) on chromosome or gene function that is not accompanied by a change in DNA sequence” (Yoder et al., 1997). These include DNA methylation, histone modifications and non-coding RNAs, which regulate appropriate gene activation at each step of neural stem cell self-renewal or differentiation.

**DNA methylation** is an important epigenetic mechanism regulating neural stem cells during embryonic development. It is catalyzed by a family of DNA methyltransferases including Dnmt1 (a maintenance methyltransferase) and Dnmt3a, and Dnmt3b (*de novo* methyltransferases) at CpG dinucleotides of genomic DNA throughout developing tissues, as well as in the adult (reviewed in Jaenisch & Bird, 2003). Disruption of DNA methyltransferase genes in mice leads to drastic demethylation of the genome and mid-gestational lethality. Mutations in genes encoding proteins that regulate DNA methylation have been linked to human neurological disorders including Rett, Fragile-X, and ATRX (a-thalassaemia mental retardation) syndromes (Robertson & Wolffe, 2000), suggesting that DNA methylation is crucial to brain development and function.

Dnmt1 is highly expressed in the developing nervous system, and maintains DNA methylation in dividing neural precursors (Goto et al., 1994). Furthermore, the *de novo* methyltransferases Dnmt3a and Dnmt3b exhibit complementary expression patterns in neural precursors, where Dnmt3b is mainly expressed in neural precursors during the period of
neurogenesis, while Dnmt3a expression predominates in late gestation and in the adult (Feng et al., 2005; Li et al., 1992), suggesting that the de novo methyltransferases play different roles during neural precursor differentiation.

How does DNA methylation mediate gene regulation? This occurs via at least two mechanisms. First, methylation of a CpG motif within a transcription factor-binding element can directly inhibit the binding of some transcription factors to their target sequence. This is the case for STAT3 binding to the gfap promoter (Tazikawa et al., 2001), as will be discussed shortly. Second, in a more general mechanism, methyl-CpG-binding domain (MBD)-containing protein family members such as MeCP2 and MBD1 bind methylated CpG motifs and suppress gene expression by acting as transcriptional repressors. The MBD proteins can also recruit other corepressor proteins and histone modification enzymes, leading to chromatin remodeling and gene silencing (Jones et al., 1998; Nan et al., 1998; Fuks et al., 2003).

Recent studies suggest that the methylation status of astrocyte-specific gene promoters is a critical intrinsic determinant for regulating the timing of gliogenesis. This idea largely stemmed from the observation that E14 precursors cultured in gliogenic conditions are more responsive to gliogenic stimuli than E11 precursors, and have increased potential to generate GFAP-positive astrocytes (Molné et al. 2000; Takizawa et al., 2001; He et al., 2005). Analysis of the methylation status of the gfap, and subsequently the s100b, promoters in these two populations of precursors, revealed that they are hypermethylated in early populations of precursors, and are barely methylated in the E14 precursors (Takizawa et al., 2001; Namihira et al., 2004). Specifically, CpG methylation occurs within the STA3-binding element (TTCGGAGAA) within the gfap promoter, and inhibits STAT3 binding at this target sequence (Takizawa et al., 2001). Thus, even though JAK-STAT signaling is active in early precursors, it can not effectively induce astrocyte-specific genes due to DNA methylation, thereby contributing to the regulation of the gliogenic switch.

Consistent with these findings, conditional disruption of dnmt1 in precursors in vivo results in decreased neurogenesis and precocious gliogenesis (Fan et al., 2005). In this case, in addition to aberrant upregulation of gfap and s100b expression, genes involved in gp130-JAK-STAT signaling, such as STAT1, were also derepressed, which was attributed to the
demethylation of these gene’s promoters as well. It was subsequently shown that gliogenic cytokines themselves can derepress this pathway (He et al., 2005), providing an extrinsic positive-feedback loop to regulate demethylation and activation of this gliogenic pathway. Altogether, these data support a model where DNA methylation regulates the timing and magnitude of astrocyte differentiation through modulation of JAK-STAT activation, as well as direct regulation of astrocyte-specific genes.

But how do precursors at late gestation, which are competent to generate astrocytes, still generate neurons without activating astrocytic programs in response to cytokines? Or put differently, how are gliogenic programs suppressed in neurons? MBD proteins, which are predominantly expressed in neurons (Setoguchi et al., 2006) may play an important role in this repression of gliogenic fate. MBD1-deficient precursors generate fewer neurons that wildtype counterparts (Zhao et al., 2003; Li et al., 2008), and ectopic expression of MBDs inhibits the expression of astrocytic genes in precursors, and their differentiation into astrocytes under gliogenic conditions (Setoguchi et al., 2006). Although precursors lose methylation at the STAT3 binding site within the *gfap* promoter in late neurogenesis, other regions near the transcription start site of this gene remain highly methylated in precursors as well as in their neuronal progeny. MeCP2 binds to this highly methylated region in neurons, as well as to hypermethylated CpG sites near the transcription start site of the *s100b* gene, to suppress their expression. The dominant-negative bHLH *Id1-4* genes are also targets of MECP2-mediated repression during neuronal differentiation (Peddada et al., 2006). Thus, regulation of region-specific DNA methylation and MBD protein expression likely plays an important role in maintaining precursor and progeny identity. Nevertheless, many questions remain to be answered. How is DNA methylation removed? How are DNA methyltransferases regulated in specific cell types? Are neurogenic gene promoters also methylated in precursors and astrocytes?

Another major epigenetic mechanism which regulates the accessibility of transcription factors to their target sequence in neural precursors is the modulation of chromatin structure by histone modification. The best studied forms of histone modifications include histone acetylation and histone methylation.
The acetylation of lysine residues at the N-terminal tails of histones is catalyzed by histone acetylases (HACs), and decreases the interaction between the positively charged histone tails and the negatively charged DNA. This relaxes the nucleosomes, and results in a more open conformation of DNA, accessible to transcription factors. Histone deacetylases (HDACs) carry out the reverse reaction, and generate a transcriptionally inactive DNA state: deacetylated histones package the DNA into a more condensed chromatin, preventing access by transcription factors and thus effectively repressing transcription (reviewed in Hsieh & Gage, 2005).

HDAC1 and HDAC2 are differentially expressed in neural precursors and differentiated neural cell types (MacDonald & Roskams, 2008). However, the role of specific HDACs in neural development remains to be determined. Studies in which histone deacytelation was globally inhibited using HDAC inhibitors, suggest that they are required for stem cell maintenance, and suppression of neurogenesis. Specifically, adding an HDAC inhibitor trichostatin (TSA) to cultured neural precursors increases their differentiation into neurons, but decreases astrocyte formation (Balasubramaniyan et al. 2006). Similarly, inhibiting histone deacetylation in adult neural precursors in culture or in vivo leads to a decrease in proliferation, coupled to an increase in neuronal differentiation (Hsieh et al., 2004). The expression of the neurogenic transcription factor NeuroD was also found to be upregulated under these conditions (Hsieh et al., 2004) suggesting that histone deacetylation is required to repress neurogenic programs or that histone acetylation promotes neurogenesis. Although this appears to generally be the case in the dorsal telecephalon, inhibiting HDAC activity in precursors derived from the ganglionic eminences leads to a decrease in interneuron differentiation (Shakèd et al., 2008), suggesting that the different transcriptional codes regulating glutamatergic neurons versus GABAergic interneuron development may be differentially regulated by transcriptional activators and repressors respectively.

Chromatin modifiers such as DNA methyltransferases and HACs/HDACs are recruited to specific genomic loci by DNA binding proteins, either repressors or activators (reviewed in Peterson et al. 2004, Shi et al. 2008). As discussed in the previous section, most neurogenic and gliogenic transcription factors interact with co-activators such as CBP/p300, which possess intrinsic HAT activity, and is important for the optimal expression of target genes. As mentioned previously, in a context where CBP/p300 levels are limiting,
neurogenic and gliogenic transcription factors can compete for and can sequester CBP/p300 away from each other, thereby antagonizing the activity of transcription factors expressed at lower levels.

Many transcriptional repressors interact directly or indirectly with HDACs and other histone modification enzymes to repress transcription of their target genes. Two well-characterized examples of transcriptional repressors that regulate precursor maintenance and neurogenesis by repressing neuronal genes in neural precursors are the RE1 silencing transcription factor (REST; a.k.a neuron-restrictive silencer factor NRSF) and the polycomb group protein Bmi1.

REST is a zinc finger protein that represses neuronal genes by binding to a 23 base pair conserved motif known as RE1 (repressor element 1), found in many neuronal-specific genes (Chong et al., 1995; Schoenherr & Anderson, 1995) REST is highly expressed in embryonic stem cells as well differentiated non-neuronal cells, and has been shown to silence neuronal genes in these cell types (reviewed in Ballas & Mandel, 2005). As such, genetic perturbation of REST expression or function in the developing embryo results in ectopic expression of neuronal genes in non-neural tissues, and embryonic lethality (Chen et al., 1998). REST therefore plays a fundamental role in orchestrating the progression of pluripotent cells to lineage-restricted neural progenitors, and is thought of as a master regulator of the neuronal phenotype (reviewed in Ballas & Mandel, 2005).

It has recently been shown that REST functions to repress but not permanently silence neuronal genes in neural precursors (Ballas et al., 2005). It does so by interacting with co-repressors including Co-REST, mSin3A, MeCP2, N-CoR and the retinoic acid receptor (Ballas et al. 2005), which then recruit HDAC complexes and induce a repressive chromatin state that blocks the expression of proneural genes such as Mash1 and BDNF, calbindin, and synaptotagmin 4 (Ballas et al., 2005). As such, inhibiting HDAC activity in the experiments discussed above could promote neurogenesis, at least in part, by upregulating the expression of REST-regulated neuron-specific genes. REST itself is regulated at the transcriptional level in neurons, and posttranslationally in neural precursors (Ballas et al., 2005). Thus, REST expression regulation, in combination with histone modifications and specific REST interactions that occur at the RE1 site, regulates the extent
and reversibility of the repressive effect of REST on neuronal gene expression (reviewed in Ballas et al., 2005).

Bmi1 is another transcriptional repressor that regulates precursor maintenance and neurogenesis. Bmi1 is part of the evolutionarily conserved Polycomb Group (PcG) family of transcriptional repressors that have been implicated in regulating stem cell self-renewal, including that of neural stem cells (reviewed in Valk-Lingbeek et al., 2004). More precisely, as a polycomb repressive complex 1 (PRC1) proteins, it is implicated in preserving gene repression patterns (set by other PcGs of the PRC2 type) during cell division to maintain cellular identity. It recognizes a H3 lysine 27 mark initially set by PRC2 enzymes (Czermin et al. 2002), but its precise mode of action in vivo is not known. In vitro, PRC1 type PcGs interact with histones and histone methyltransferases, and counteract SWI/SNF-chromatin-remodeling complexes which promote transcriptional activation (Breiling et al., 1999; Levine et al., 2002; Ogawa et al., 2002; Sewalt et al., 2002). These repressive complexes may work as they do in Drosophila, where PcGs appear to inhibit the transcription initiation machinery (Dellino et al., 2004; Wang et al., 2004).

Bmi1 is expressed in the neural stem cells and precursors, but not in differentiated cells (Zencak et al., 2005). Targeted deletion of bmi1 in mice leads to progressive postnatal growth retardation and neurological defects (van der Lugt et al. 1994), and to decreased proliferation in the newborn cortex and adult subventricular zone (Zencak et al., 2005). By unclear mechanisms, bmi1-/- mice also show increased astrocyte formation at birth and develop generalized gliosis at one month (Zencak et al., 2005).

Evidence from studies where bmi1-deficient neural stem cells were cultured and passaged as neurospheres suggests that Bmi1 is required for the self-renewal of neural precursors (Molofsky et al., 2003). In these studies there were fewer and smaller neurospheres generated from bmi1-deficient precursors. Furthermore, it appears that embryonic neural stem cells are less dependent on Bmi1 for their self-renewal, whereas Bmi1 deficiency leads to depletion of postnatal precursors (Molofsky et al., 2003; Fasano et al., 2007), suggesting that other PcGs might compensate for bmi1 deficiency during embryonic cortical development. Acute knockdown of bmi1 leads to a cell-autonomous decrease in precursor proliferation (Zencak et al., 2005; Fasano et al., 2007). The cyclin-dependent kinase inhibitor gene p16^{ink4a} and p19^{arf}, as well as p21, are upregulated in the absence or
acute knockdown of bmi1, leading to repression of cell cycle progression (Molofsky et al., 2003; Bruggeman et al., 2005; Fasano et al., 2007), which can be rescued in settings where these regulators are absent or reduced. Bmi1 thus promotes neural stem cell self-renewal and proliferation by acting as a potent repressor of the cell cycle inhibitors p16\textsuperscript{ink4a}, p19\textsuperscript{arf}, and p21, important regulators of pRB and p53 pathways (reviewed in Sharpless & DePinho, 1999; Sharpless, 2005; Xiong et al., 1993; Bartek et al., 1996).

This brings us to another important group of intrinsic factors regulating fate determination of cortical precursors, the cell cycle regulators.

f) cell cycle regulators

The notion of symmetric versus asymmetric divisions and polarized distribution of fate determinants during mitosis that was discussed at the beginning of this chapter cannot account for all fate decision mechanisms. Some investigators have proposed that, following mitosis, the length of time that a daughter cell is exposed to fate determinants and to environmental cues could also influence its fate. Thereby, the length of time a precursor spends in each phase of the cell cycle could be a permissive factor in regulating fate. Interestingly, increases in the lengthening of the G1 phase have been observed in cycling precursors at the onset of neurogenesis \textit{in vivo} (Takahashi et al., 1993; 1995; Caviness et al., 1995), a time where proliferative (symmetric) divisions switch to neurogenic (asymmetric) divisions in the developing ventricular zone, suggesting that neurogenic factors and cues could have more time to effectively act on fate determination during the period of neurogenesis. Similarly, signals which promote neurogenesis might do so by altering cell cycle kinetics, and lengthening the cell cycle to permit neurogenic intrinsic mechanisms to accumulate, localize properly, and carry out their functions. Thus, a link between cell cycle kinetics and asymmetric divisions can be made.

To investigate whether altering cell cycle length, in particular G1 length, could cause changes in neurogenesis, cortical precursors have been treated with various pharmacological inhibitors of cyclin-dependent kinases and G1 progression, which lengthen but do not arrest the cell cycle. Experiments in which cell cycle dynamics of neuroepithelial precursors are altered have shown that slowing or stopping the cell cycle by lengthening G1 phase is sufficient to increase neuronal differentiation (Calegari & Hutner, 2003; Calegari et al.,
The expression of TIS21, an inhibitor of G1 progression (Matsuda et al., 2001; Tirone et al., 2001), is restricted to neuron-generating, but not proliferating, precursors (Iacopetti et al., 1999) suggesting that it could functionally promote neurogenesis by lengthening the cell cycle or inhibiting G1 to S-phase transition (Calegari & Huttner, 2003; Canzoniere et al., 2004). Alternatively, it could regulate cell cycle exit, leading to differentiation.

During cortical development, cell cycle exit and neuronal differentiation are tightly coupled, and factors that regulate cell cycle exit promote neurogenesis. The cell cycle is divided in four stages: Gap1 (G1), Synthesis of DNA (S-phase), Gap2 (G2) and Mitosis (M). Progression through each of these stages is controlled by distinct cyclins and cyclin-dependent kinases (CDKs), as well as two cyclin-dependent kinase inhibitors (CKIs) families including the Ink4a family which includes p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c}, p19^{Ink4d/arf}, and the Kip/Cip family which includes p21^{Cip1}, p27^{kip1} and p57^{kip2}. Between G1 and S-phase, a newborn cell proceeds through a G1 checkpoint, where mitogenic or anti-mitogenic signals determine whether it will proceed to S-phase and initiate a new division or exit the cell cycle into G0 or G1 arrest, where it will either adopt a quiescent or differentiated state (reviewed in Paris & Andrisani et al., 2007).

Cell cycle regulators thus play important roles in regulating the balance between self-renewal and differentiation, and as mentioned previously, many transcriptional regulators of self-renewal and neurogenesis act in part by interfering or cooperating with cell cycle proteins, a recurring example being pRb and CKIs.

The retinoblastoma protein (pRb) is known as a tumor suppressor, and functions by controlling cell cycle progression from G1 to S-phase (Weinberg et al., 1995). pRB is regulated by phosphorylation, in response to mitogenic stimuli. In its hypophosphorylated state, pRB prevents premature S-phase entry by binding to and inhibiting the E2F family of transcription factors, actively repressing their target genes. Upon stimulation for cell cycle re-entry, pRB is phosphorylated by cyclinD-cdk4 and cyclin-E-cdk2 complexes, reducing its affinity for E2Fs and relieving its repression at target genes (Sears & Nevins, 2002). pRb has been associated with neuronal differentiation (Slack et al., 1993), and inactivation of pRb results in early embryonic lethality and hematopoietic and neurological defects attributed to delay or failure of differentiating cells such as neuronal precursors to permanently withdraw
from the cell cycle and their aberrant re-entry into S-phase, leading to neuronal apoptosis (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992; Slack et al., 1998; Callaghan et al., 1999; Ferguson et al., 2002).

CKIs mainly repress proliferation by interfering with CDK-mediated pRb phosphorylation, and appear to regulate the timing of neurogenesis by controlling cell cycle length and probability of cell cycle re-entry. For example, inactivation of the CKI p27kip1, results in decreased production of early born neurons but increases the production of later-born neurons, causing an enlargement of the upper cortical layers (Goto et al., 2004), suggesting that p27kip1 controls the birth date of cortical neurons. Conversely, the overexpression of p27kip1 reduces upper-layer neuron production (Tarui et al., 2005). p27kip1 also regulates neuronal differentiation by interacting and stabilizing the neurogenic bHLH Ngn2 (Nguyen et al., 2006).

Thus intrinsic mechanisms, as do extracellular signals, act in concert with cell cycle regulators to determine neural precursor fate.

**Bii. Extracellular signals regulating cortical development**

The impact of intrinsic fate determinants (discussed previously) on cell fate is ultimately regulated by an interplay of pleiotropic environmental signals, often regulating stem cell maintenance and proliferation, neurogenesis or gliogenesis in a spatiotemporal and cell context-specific manner (Illustrated in Figure 1.2).

**a) Extracellular signals regulating neural stem cell maintenance and proliferation**

a) i. **Notch signaling promotes radial precursor maintenance and self-renewal while inhibiting neurogenesis**

Notch signaling is perhaps the most extensively studied extracellular signaling modality in the context of stem cell fate regulation in many model systems (reviewed in Chiba, 2006), including embryonic neural stem cell fate determination (reviewed in Louvi & Artavanis-Tsakonas, 2006). Cumulative evidence from many studies points to an active role for Notch signaling in promoting stem cell self-renewal and radial precursor identity, and in inhibiting neurogenesis, but not astrogliogenesis. The nature of the studies that will be presented in this section also best exemplifies the cell context-dependent effects common
to many signaling pathways in determining neural precursor fate outcomes, and as such are worthy of close examination.

Notch signaling is activated when transmembrane Notch ligands including Delta or Jagged from one cell bind to and activate a neighboring precursor’s transmembrane Notch receptor. Upon activation, the Notch intra-cellular domain (NICD) is cleaved by the \( \gamma \)-secretase complex, and translocates to the nucleus, where it can interact with the DNA binding protein RBP-J (also known as CBF1, CSL, Lag-1 and Su(H)) to activate target genes such as the inhibitory bHLHs \( \text{hes1} \) and \( \text{hes5} \) (Kageyama & Nakanishi, 1997), and \( \text{blbp} \) (Patten et al., 2003; 2006) whose regulatory regions contain RBP-J binding sites (Jarriault et al., 1995; Anthony et al., 2005). In the absence of Notch signaling, RBP-J interacts with co-repressors such as N-CoR to repress Notch-target and other genes (Hermanson et al., 2002; reviewed in Lai, 2002). Interestingly, RBP-J binding sites have also been identified in the \( \text{g} \)\text{fap} promoter, suggesting that it is either a target of Notch signaling (Ge et al., 2002) and/or of RBP-J/N-CoR-mediated repression (Hermanson et al., 2002). Non-canonical Notch signaling can also occur independently of RBP-J, where another Notch signaling effector, deltex1 (DTX), interacts with the NICD (Yamamoto et al., 2001) and promotes transcription of other genes such as \( \text{erbb2} \) (Patten et al., 2003; Patten et al., 2006), which regulates radial precursor identity and astrocyte formation (Schmid et al., 2003).

Three of the four vertebrate Notch family members, Notch1, Notch2, and Notch3 are expressed in neural precursor cells in the VZ/SVZ of the developing rodent CNS (Higuchi et al., 1995; Prakash et al., 2002) along with the Notch ligand Delta and Jagged (Lindsell et al., 1996). While \( \text{Notch1} \)-deficient mice die in mid-gestation and exhibit mild defects in early cortical development, the \( \text{Notch2} \) and \( \text{Notch3} \)-deficient mice do not (de la Pompa et al., 1997; Hamada et al., 1999; Krebs et al., 2003; reviewed in Yoon & Gaiano, 2005), suggesting that Notch1 is the major player in CNS development. As such, in contrast to Notch1 signaling, the role of Notch2 and Notch3 in neural development has not been well studied, and studies in double or triple conditional knockouts have yet to be published.

While Notch receptors are apparently ubiquitously expressed in the VZ/SVZ, Notch1 activation does not occur uniformly in these proliferative zones. Results from studies where Notch activation was measured in fixed cortical sections by immuno-detection of the cleaved form of NICD-1 (Tokunaga et al., 2004) or live by detection of transgenic or
Transient canonical Notch signaling-responsive fluorescent gene reporters reflecting Notch activation (Kohyama et al., 2005; Hansson et al., 2006; Mizutani et al, 2007), suggest that Notch1 signaling is only active in a subset of precursors. Active Notch1 signaling is selectively observed in radial precursors during embryonic development (Tokunaga et al., 2004; Kohyama et al., 2005), while it is excluded from intermediate neuronal precursor or early differentiating neurons (Tokunaga et al., 2004; Mizutani et al., 2007). Consistent with these findings, Notch1 has previously been shown to segregate asymmetrically to radial precursor daughter cells during asymmetric divisions (Chenn & McConnell, 1995) and acute knockdown of RBP-J promotes the conversion of ventricular zone precursors into intermediate/basal precursors (Mizutani et al., 2007). The reverse ectopic expression of RBP-J is however insufficient in converting intermediate precursors back to a neural stem cell phenotype. A transient activation of Notch signaling was also detected in astrocyte-generating precursors during perinatal development (Tokunaga et al., 2004; Kohyama et al., 2005), altogether suggesting that Notch signaling regulates multiple aspects of fate specification during cortical development.

Inactivating mutations in key components of the murine Notch signaling pathway including Notch receptors (de la Pompa et al., 1997; Hitoshi et al., 2002, Lütolf et al., 2002; Yoon et al., 2004; Yang et al., 2004), or the Notch ligands such as Delta-like-1 (Dll1) (Yun et al., 2002; Grandbarbe et al., 2003), or effectors such as RBP-J (Hitoshi et al., 2002; Oka et al., 1995; de la Pompa et al., 1997), target genes hes and herp (Ishibashi et al., 1995; Ohtsuka et al., 1999; Ohtsuka et al., 2001; Nakamura et al., 2000; Hatakeyama et al., 2004; Sakamoto et al., 2003), and modulators such as y-secretase complex proteins presinilin 1 and 2 (PS1 and PS2) (Hitoshi et al., 2002; Donoviel et al., 1999; Handler et al., 2000), have revealed a requirement for Notch signaling in the maintenance of self-renewal of neural stem cells in culture and in vivo. Conditional inactivation of Notch1 in a Notch3-deficient mouse background furthermore results in decreased CNS blbp expression (Anthony et al., 2005), suggesting that Notch signaling plays an important role in regulating radial precursor morphology and differentiation. In Drosophila, another modulator of Notch signaling, Numb is thought to antagonize Notch signaling (reviewed in Roegiers & Jan, 2004), but the role of Numb and Numblike in vertebrates is still unclear. Inactivation of the Notch modulators Numb and Numblike leads to contradicting conclusions with regards to radial
precursor biology (Petersen et al., 2002; Petersen et al., 2004; Li et al., 2003), suggesting that they may act antagonistically or in parallel with Notch in a spatiotemporal manner (reviewed in Yoon & Gaiano, 2005). In general however, mutations which inhibit or decrease Notch signaling in neural precursors lead to decreased self-renewal as assessed by neurosphere assays, as well as depletion of radial precursors and precocious neuronal differentiation \textit{in vivo} (reviewed in Yoon & Gaiano, 2005), suggesting that Notch actively promotes radial precursor identity and self-renewal, and inhibits neurogenesis. Evidence also suggests that a role for Notch signaling precedes its role in inhibiting neuronal differentiation, where it also acts in regulating the generation of precursor diversity in the developing brain (Gaiano et al., 2000; Grandbarbe et al., 2003). Beyond cell fate specification, Notch signaling also regulates neuronal maturation (Redmond et al., 2000; Sestan et al., 1999; Franklin et al., 1999; Klein et al, 2004; Huang et al., 2005) and neuronal function in learning and memory in the adult brain (Costa et al., 2003; Yu et al., 2001; Saura et al., 2004; Wang et al., 2004)

The pleiotropic effects of Notch signaling on neural stem cell fate determination are highly dependent on other signaling pathways, which are temporally and spatially regulated (as will be discussed in the next sections). These context-dependent effects of Notch signaling are best illustrated by studies in which the transient ectopic expression of a constitutively active form of Notch (caNotch) results in different fate outcomes based on the timing of manipulation during cortical precursor development. Transfection of caNotch in early cortical precursors \textit{in vitro} or \textit{in vivo}, promotes radial precursor proliferation and inhibits neurogenesis (Nye et al., 1994; Saito & Nakatsuji, 2001; Mizutani & Saito, 2005) as well as oligodendrocyte formation (Wang et al., 1998; Gaiano et al., 2000) but not astrocyte formation, which is typically increased in response to Notch activation (Nye et al., 1994; Gaiano et al., 2000; Chambers et al., 2001). Perinatally, this early activation of Notch leads to an ultimate decrease in neuronal numbers and concomitant increase in astrocyte formation and potential adult stem cells (Gaiano et al., 2000; Gaiano & Fishell, 2002). Thus, it is clear that neuronal fates are inhibited by Notch activation, while astroglial fates are, at least, permissively promoted in this context (Nye et al., 1994; Gaiano et al., 2000). Nonetheless, evidence from a recent study where Notch signaling was temporarily activated in ventricular zone precursors suggests that activating Notch signaling in early precursors also contributes to the progressive restriction of neuronal fates in precursors, without the generation of an
alternative astrogliogenic fate. For example, if caNotch is transfected in E13.5 precursors, but turned off at E15.5, the affected precursors which were initially maintained as proliferating radial precursors will generate later-born neurons, even when transplanted in the early cortex (Mizutani & Saito, 2005). Therefore, although Notch signaling instructively promotes self-renewal and inhibits neurogenesis in radial precursors by regulating the various Notch-target genes discussed previously, the occurrence of alternative late-neuronal fates or astrocytic fates are most likely regulated in a permissive/modulatory fashion, and are ultimately determined by the timing of Notch activation and by the amount of additional cell cycles undergone by the genetically manipulated precursors. Interestingly, while embryonic activation of Notch leads to increased astrogliogenesis, persistent ectopic activation of Notch signaling after birth inhibits the differentiation of GFAP-positive astrocytes (Kohyama et al., 2005), suggesting that Notch signaling may inhibit differentiation in general, depending on the timing of its activation. Consistently, while activation of Notch signaling in precursors in the dorsal cortex results in increased astrocyte formation at the expense of neurogenesis, at the same time point it causes precursors from the olfactory bulb to become quiescent and to fail to differentiate (Chambers, 2001). Although the role of Notch signaling in regulating gliogenesis remains unclear, it is possible that it promotes astrocyte formation and \textit{gfap} expression by relieving the potential RBP-J/N-CoR-mediated negative regulation at this promoter (Hermanson et al., 2002). Altogether, these studies best illustrate the complexity and context-dependent effects of manipulating signaling pathways in cortical precursors; something that must be kept in mind when performing or critically evaluating similar studies.

Although much emphasis has been placed on understanding the regulation of Notch signaling in the signal-receiving radial precursors, not much is known about the signal-generating cells, and how neighboring precursors interact to regulate self-renewal versus differentiation. It has been shown that the Notch ligands Delta-like -1 and -3 and Jagged are not expressed by radial precursors, but rather by undifferentiated cells adjacent to them (Campos et al., 2001), suggesting that radial precursor daughter cells do not typically send Notch signals and regulate each other. A more recent study has revealed that Notch signaling in radial precursors is generated by newly-born neurons as well as intermediate precursors that are adjacent to radial precursors upon their original production (Yoon et al., 2008). For example, Mind bomb (Mib), a RING-type E3 ubiquitin ligase which regulates the
endocytosis and thereby availability of all of the canonical Notch ligands (Dll-1-3,-4; Jagged-1,-2) in vertebrates (Koo et al., 2005, 2007), is expressed in young neurons as well as intermediate precursors, but not radial precursors (Yoon et al., 2008). Conditional inactivation of Mib in the developing forebrain leads to the complete abrogation of Notch signaling and radial precursor maintenance, and to their premature differentiation to intermediate precursors, and finally neurons (Yoon et al., 2008), suggesting that Mib-expressing IPCs and newly-born neurons are responsible for the majority of Notch signaling during cortical development. In these experiments, the mechanism of radial precursor depletion was thought to occur by an increase in abnormal symmetrical divisions yielding either two intermediate precursors or two neurons at the expense of asymmetric divisions that ensure the self-renewal and stemness of radial precursors, and was due to defective Notch signaling between intermediate precursors or newly-born neurons and radial precursors during the phase of neurogenesis. Furthermore, mutations in molecules that regulate the interaction between radial precursors and the neurons that migrate along their radial scaffold, also affect radial precursor morphology and differentiation (Forster et al., 2002; Kodowaki et al., 2007), suggesting that the interactions between radial precursors and their progeny are important for their maintenance.

As mentioned above, the effects of Notch signaling are reliant on its integration with other developmental signaling pathways such as the Wnts, BMP, FGF and cytokine signaling in drosophila and vertebrates (Sahlgren & Lendahl, 2006). The integration of multiple signals, converging on the intrinsic mechanisms discussed earlier, determines the response of precursors. For example, Notch appears to modulate the responsiveness of cultured precursors to FGF signaling (Yoon et al. 2004), which is the most relevant mitogenic pathway in neural precursors (reviewed in Mason, 2007), as will be discussed next.

a) ii. Fibroblast growth factor (FGF) signaling regulates precursor proliferation

The mammalian fibroblast growth factor (FGF) family includes at least 22 members, some of which, along with other important morphogens such as Wnts, Shh, and BMPs, play important roles at multiple steps of cortical development, including neural induction, regional specification and patterning of the cerebral cortex, as well as cell fate determination through
regulation of proliferation, differentiation and survival (reviewed in Mason, 2007). FGF ligands bind to one of four tyrosine kinase receptors (FGFR1-4) with different affinities based on alternative splicing of both ligands and receptors and binding of the obligate co-factor heparate sulfate proteoglycans (HSPGs). Ligand binding in a 2:2:1 FGF:FGFR:HSPG ratio causes receptor dimerization and the resulting auto-phosphorylation of the receptors leads to activation of two major phosphorylation cascades: the Ras/MEK/ERK mitogen activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI-3K)/AKT pathways. These cascades are activated via recruitment of various adaptor proteins such as FRS2 and Gbr2/Gab1 to the phosphorylated receptors. Other enzymes/adaptor proteins including PLC-γ, Src, Shc and Crk can also be recruited to the receptors, inducing other signaling cascades such as Ras-Rac-RhoGTPase signaling, or impinging on the other MAPK protein p38MAPK and Jun kinase in a cell-context specific manner (reviewed in Mason, 2007; Illustrated in Figure 1.4). The MAPK proteins induce transcriptional events by phosphorylating various transcription factors, thereby influencing cellular responses such as proliferation and differentiation, while RhoGTPases regulate cytoskeletal dynamics.

While multiple FGF ligands and receptors are expressed in defined regions of the neural tube and cortical neuroepithelium during regional patterning of the cortex, their individual and redundant roles and differential abilities to activate FGF signaling remain unclear, along with the identity of the relevant FGF ligands/receptors that regulate neural stem cells. Most of the studies on FGF signaling and neural cell fates focus on FGF2 (or basic fibroblast growth factor), which is widely expressed in the embryonic CNS (reviewed in Baird, 1994), can be detected outside cells in the ventricular zone (Weise et al. 1993) and has long been accepted as a mitogenic factor for cultured CNS precursors (reviewed in Temple & Qian, 1995; Rao et al., 1999). Interestingly, Fgf2 transcripts are detected as early as E9 in the murine ventricular zone (Nurcombe et al., 1993), while both mRNA and protein levels increase dramatically from E14-E18, and drop postnatally (Powell et al., 1991a; Giordano et al., 1992; Weise et al., 1993; Vaccarino et al., 1999; Raballo et al., 2000). Fgf1 mRNA is also detected from E11.5 (Nurcombe et al., 1993). Mirroring the expression pattern of FGF2, specific isoforms of FGFR1, FGFR2 and FGFR3 mRNA can be detected in the neuroepithelium between E10-E14 (Qian et al., 1997), while FGFR1 & 2 protein expression also drops around E18 (Vaccarino et al., 1999; Raballo et al., 2000), an expression pattern
which coincides with the peak of neurogenesis and early stages of gliogenesis; or from an alternate perspective, a time when neural stem cell proliferation decreases dramatically. The presence of these ligands and receptors in the early neuroepithelium suggests that changes in FGF signaling could regulate precursor proliferation, survival, and/or differentiation in an autocrine or paracrine manner. Interestingly, at the peak of neurogenesis, FGF2 and FGFR1 are expressed throughout the VZ, with strongest expression at the end feet of apically localized precursors. By E17.5, only a few scattered apical cells show strong expression (Raballo et al., 2000). The identity of these cells has not been examined, but it would be interesting to determine if these correspond to a few remaining multipotent stem cells at this stage of cortical development, and how other precursors lose FGFR expression.

FGF signaling is absolutely required for the expansion of neural stem cells in culture (Qian et al., 1997; reviewed in Rao et al., 1999) as well as in vivo. Fgf2 knockout mice (Zhou et al., 1998) show normal organogenesis with the exception of the cerebral cortex (Dono et al., 1998; Ortega et al., 1998; Vaccarino et al., 1999), where E10.5 fgf2-/- embryos are missing half of their wildtype counterpart’s dorsal neural stem cell population at the onset of neurogenesis, leading to decreased generation of both neuronal and glial progeny in the mature brain (Vaccarino et al., 1999; Raballo et al., 2000; Korada et al., 2002). FGF2 signaling does not appear to be required for survival of the precursors in vivo, but is required for their proliferation (Vaccarino et al., 1999; Raballo et al., 2000). Similar phenotypes are observed when a kinase-dead form of FGFR1 is expressed in neural precursors (Shin et al., 2004), suggesting that FGF-FGFR1-mediated signaling regulates neural precursor expansion and, indirectly, neurogenesis and gliogenesis. To circumvent the early proliferation defects in FGF signaling-deficient mouse models, acute knockdown of FGFR1 and FGFR3 receptors was performed in sort-purified populations of E13 neural stem cells. In this system, inactivation of FGFR1 and 3 decreased neural stem cell proliferation and promoted their differentiation into neurons (Maric et al., 2007), suggesting that the impaired neurogenesis in the FGF2-/- mice may reflect both an initial reduction in the founder precursor pool as was initially interpreted, but may also be attributed to a further depletion of the precursor pool caused by premature neurogenesis and asymmetric division of the remaining precursors. Consistently, total cell cycle time is not affected in fgf2-deficient precursors (Vaccarino et al., 1999), an observation that was, however, disputed in subsequent studies (Lukaszewicz et al., 2002).
Furthermore, activation of FGFR1 and 3 in the same isolated precursors leads to increased self-renewal via symmetrical divisions (Maric et al., 2007). Consistent with these findings, mice heterozygous for the constitutively active K644E kinase domain mutation in FGFR3 (Iwata et al., 2000;2001) exhibit a marked enlargement of the brain, and increased neuroepithelial and cortical thickness throughout development (Inglis-Broadgate et al., 2005) due to increased proliferation and decreased apoptosis of neural precursors throughout the CNS (Thomson et al., 2007), a phenotype that could be rescued in E11 cultured precursors by inhibiting MEK, but not in precursors cultured between E12-15 (Thomson et al., 2007), suggesting that at different stages, multiple signaling cascades promote proliferation in response to FGF activation. Together, these data further indicate that FGF2-FGFR1/3 signaling promotes symmetrical divisions of neural precursors and regulates cell numbers in the developing cortex. However, other mitogenic factors analogous to FGF2 also more than likely play a role in this process. For example, other family members Fgf8, Fgf15, Fgf17 and Fgf18 are also expressed in the rostral midline of the early forebrain (Crossley et al., 1995; Maruoka et al., 1998), suggesting that they could also regulate neural precursor fate determination.

Culture experiments also suggest that different types of precursors may be regulated differently by FGF signals in a dosage-dependent fashion. For example at low FGF2 concentrations, cultured precursors are permitted to generate neurons, while high FGF2 concentration (10-40ug/ml) cause increased proliferation and increased oligodendrocyte formation or, in the presence of exogenous factor such as IL-6 related cytokines, increased astrocyte formation (Qian et al., 1997), suggesting that FGF regulates glial lineage-restricted precursors in this setting, without affecting neurogenesis. On this note, FGF2 does not, on its own, promote astrocyte formation in cultured precursors, but it influences cytokine-induced astrocyte formation, at least in part, by promoting an active chromatin conformation at the gfap promoter (Song & Ghosh, 2004). However, intra-ventricular injection of FGF2 in the E15.5 mouse brain leads to increased proliferation and neurogenesis, without affecting astrocyte formation; while injecting FGF2 in the E20.5 mouse brain increases astrocyte formation (Vaccarino et al. 1999). This response to a transient FGF2 exposure was interpreted as being caused by increased number of cell cycles undertaken by the precursors which ultimately generated increased numbers of neurons and astrocytes respectively.
Therefore, FGF concentrations and timing of FGF stimulation likely determines precursor response.

In culture, FGF2 promotes the proliferation of precursors isolated from many CNS regions at many developmental timepoints (reviewed in Rao et al., 1999; Gritti et al., 1999; Lukaszewicz et al., 2002; Kelly et al., 2003). Exogenous addition of FGF1, FGF2 or FGF8 to E10 precursor cultures, increases proliferation and can promote or inhibit neuronal differentiation (Murphy et al., 1990; Ghosh & Greenberg, 1995; Vaccarino et al., 1999; Faux et al., 2001; Borello et al., 2008). How could FGF signaling promote proliferation at the expense of neurogenesis? PI-3K and ERK1/2 pathway activation are both required for FGF-2 induced precursor cell proliferation in culture (Learish et al., 2000; Jin et al., 2005; Shimizu et al., 2008). In some cellular contexts, FGF1 and FGF2 may inhibit neurogenesis via the Notch-Delta pathway (Faux et al., 2001). Adding FGF1 or FGF2 to cultured precursors causes an increase in Notch1 and Notch3 expression and decreases the expression of Delta 1, which were shown, as discussed above, to inhibit neurogenesis. This effect is potentiated by serum, suggesting that other factors may also function through Notch (Faux et al., 2001). Consistently, the inhibition of neuronal differentiation caused by FGF2 could be overcome by downregulating Notch1 or by disrupting Notch activation in presenilin-deficient precursors (Faux et al., 2001). Interestingly, in the absence of FGF (or analogous mitogen) neurospheres can not be generated, even in the presence of constitutively active Notch1 signaling, suggesting that Notch1 signaling-mediated proliferation and self-renewal is FGF signaling-dependent, at least in this assay. On the other hand, constitutive activation of Notch1 in the presence of FGF2 increases the efficiency of neurosphere formation, suggesting that Notch1 signaling potentiates the proliferative responsiveness of precursors to FGF2 (Yoon et al., 2004). Altogether, these results suggest that an important genetic interaction exists between FGF and Notch signaling in neural precursors, and warrants further study.

a) iii. EGF signaling promotes the proliferation of late embryonic precursors and their differentiation into astrocytes

While FGF-responsive mouse neural stem cells can be isolated as early as embryonic day 8.5, another population of EGF-responsive precursors (that proliferate in response to
EGF), arises later in cortical development (Kilpatrick & Bartlett, 1995; Tropepe et al., 1999). A population of EGF-responsive precursors also remains in the adult brain SVZ (Reynolds & Weiss, 1992, 1996; Reynolds et al., 1992). Furthermore, cortical precursors are not mitotically responsive to TGF\(\alpha\) prior to E18 (Kilpatrick & Bartlett, 1993; Ferri et al., 1996; Burrows et al., 1997), a time point coinciding with the start of astrogliogenesis. Early precursors may not be responsive to EGF ligands due to low expression levels of EGFRs or due to modulation of EGFR signaling in some way during early cortical development, leading to diverse responses to EGF at different time points of development. Consistent with the first of these two suggestions, EGFR mRNA and protein levels increase in the subventricular zone during late cortical development (Seroogy et al., 1995; Eagleson et al., 1996; Kornblum et al., 1997; Burrows et al., 1997; Caric et al., 2001). Adding FGF2 and EGF to cultured precursors at this time point produces an additive proliferative effect, suggesting that these growth factors probably regulate the proliferation of two separate, but lineage-related populations of precursors (Tropepe et al., 1999). Consistent with the idea that precursors change their competence over time, and that EGF-responsiveness does not reflect the appearance of a different precursor lineage, the developmental change in EGFR expression has been found to be regulated by extrinsic factors such as FGF2, which induces EGFR expression (Lillien & Raphael, 2000).

The EGF receptor (EGFR) is a member of the ErbB receptor family of tyrosine kinase receptors (also known as HER in humans), which includes four members: EGFR or erbB1, erbB2 (a.k.a. neu), erbB3, and erbB4. These receptors consist of a glycosylated extracellular domain that mediates ligand binding, a short hydrophobic transmembrane region, and a highly conserved large intracellular tyrosine kinase domain. EGF family members can interact through the heterodimerization of their cognate receptors, resulting in specific and diversified signaling and cellular responses (reviewed in Junier, 2000).

EGF family members can be subdivided into two groups, according to their capability to bind EGFR or not. The first group includes EGF and TGF\(\alpha\) (as well amphiregulin, heparin binding-EGF or HB-EGF, betacellulin, and epieregulin) which bind EGFR. The second group, which does not bind to EGFR, includes neuregulin1 (nrg1), nrg2, nrg3 and nrg4. Nrg1 and nrg2 are ligands for ErbB3 and ErbB4, while nrg3 and 4 only bind to erbB4.
Mouse mutants defective in EGFR signaling suggest that EGFR signaling regulates maturation of cortical precursors and astrocyte formation (reviewed in Junier, 2000). First, genetically null EGFR mice show abnormally thick ventricular zones (Threadgill et al., 1995), and depending on genetic background, show delayed astrocyte development (Kornblum et al., 1998) or reduced numbers of astrocytes (Sibilia et al., 1998), suggesting that VZ precursors may not mature into gliogenic SVZ precursors in these mice. However, the precise requirement for EGFR signaling in the regulation of neural precursor proliferation and differentiation is difficult to ascertain because these phenotypes could be attributed to astrocyte differentiation, migration or survival defects (Sibilia et al., 1998; Wagner et al., 2006). Similarly, waved-1 mice which express reduced levels of TGF-α have a decreased number of astrocytes (Weickert & Blum, 1995), and show decreased subependymal adult neural precursor proliferation (Weickert & Blum, 1995; Tropepe et al., 1997). No studies investigating the effect of acute EGFR signaling inactivation during neural precursor development have been performed, but these would be informative.

However, consistent with the above results indicating a potential role for EGFR in regulating gliogenic precursors, retinal precursor cultured in low doses of TGF-α undergo more mitoses, while the introduction of extra EGFR copies in precursors promotes their differentiation towards a glial lineage (Lillien et al., 1995). Similarly, in the developing cortex, transfection of extra EGFRs in early cortical precursors (E12-E15) cultured in the presence of EGF or TGF-α promotes two responses: at low EGF or TGF-α concentrations, this promotes proliferation of the multipotent precursors whereas at higher concentrations this promotes astrocyte formation (Burrows et al., 1997). Similarly, transfection of VZ precursors with EGFRs leads to premature astrocyte formation at the expense of neuronal differentiation in cortical explants (Burrows et al., 1997; Viti et al., 2003). Adding extra EGFRs to early precursors therefore mimicks changes in competence acquired by late precursors during embryonic cortical development, and is sufficient to alter cell fates. Changes such as increased expression of EGFR receptors therefore, in part, regulate the timing of astrocyte formation in vivo, acting via different thresholds of stimulation which are determined by the combination of receptor and ligand levels.

How does EGFR signaling regulate proliferation and astrocyte formation responses in precursors? With regard to astrocyte formation, it appears that increasing EGFR expression
in early precursors confers a premature competence to generate astrocytes in the presence of gliogenic cytokines such as LIF, but not BMPs (Viti et al., 2003), suggesting that increased expression of EGFRs is one of the molecular mechanisms regulating the timing of a change in responsiveness to LIF during cortical development. Constitently, LIF- but not BMP-mediated astrocyte formation is impeded in the EGFR null precursor cultures (Viti et al., 2003). EGFR signaling may be acting by increasing STAT3 levels and efficacy of STAT3 phosphorylation by LIF (Viti et al., 2003). However, while increasing STAT3 levels is not sufficient to increase astrocyte formation in early precursors, concomitant increase in STAT3 and EGFR levels increases astrocyte formation (Viti et al., 2003), suggesting that EGFR signaling regulates aspects of astrocyte formation downstream of phosphoSTAT3. Whether this relates to altered epigenetic regulation of the \textit{gfap} promoter akin to the action of FGF2 signaling (Song & Ghosh, 2004), or to an antagonistic interaction with inhibitors of LIF signaling downstream of phosphor-STAT3, such as PIAS3 (protein inhibitor of activated STAT3) is unknown. Furthermore, the effectiveness of EGFR signaling in eliciting changes in STAT3 phosphorylation and LIF responsiveness increases with later developmental stages (Viti et al., 2003), suggesting that other factors such as expression of Ngns, C/EBPs, and SOCs molecules, also restrict astrocyte formation in the early embryonic cortex. Finally, given that EGFRs enhance proliferation in addition to promoting astrocyte formation, it is conceivable that EGFR signaling increases LIF-responsiveness by promoting the expansion of late embryonic precursors that are competent to interpret LIF as an astrocyte-inducing signal.

Increased EGFR expression can also induce astrocyte formation at the expense of oligodendrocytes in late cortical precursors (Sun et al., 2005), in an EGF ligand-dependent manner. In this study, some late embryonic precursors were found to asymmetrically segregate EGFR during mitoses in culture, where EGFR$^{\text{high}}$ precursors represented astrocytic precursors and EGFR$^{\text{low}}$ precursors represented oligodendrocyte precursors. As such, overexpression of EGFR prevents precursors from effectively segregating EGFRs during mitoses, pushing more precursors into the astrocytic lineage (Sun et al., 2005). This study also exemplifies that precursors undergo asymmetric divisions to generate glial cells as well as neurons.
The other group of EGF family members, the neuregulins, and their cognate erbB receptors, also appear to promote precursor self-renewal but suppress gliogenesis, and will only be covered briefly here. While egfr-/- mice are viable, nrg1-/- (Meyer & Birchmeier, 1995), erbB2-/- (Lee et al., 1995), and erbB4-/- (Gassmann et al., 1995) mice die between E10 and E11, making it impossible to use these mutants to ask about the role of neuregulin signaling in cortical fate decisions. ErbB2, ErbB3 and ErbB4 transcripts and protein have been detected in the ventricular and subventricular zones as early as E11 during cortical development (Eagleson et al., 1998; Fox & Kornblum, 2005). By E16, erbB2 is present on radial precursors and developing neuronal precursors, erbB3 is expressed in cells located in ventricular zone and cortical mantle, and erbB4 is present on all cell types (Anton et al., 1997). Alternatively spliced neuregulin1 (also known as NDF, heregulin, ARIA, GGF or SMDF) transcripts are also expressed, albeit in unique patterns, in the ventricular and subventricular zones (Marchionni et al., 1993; Corfas et al., 1995; Meyer et al., 1997). The expression of at least one erbB receptor is regulated by Notch signaling. As discussed in a previous section, non-canonical Notch promotes the transcriptional activation of erbB2 (Patten et al., 2003; Patten et al., 2006), which promotes radial precursor identity and negatively regulates astrocyte formation. ErbB2-deficient radial precursors are morphologically aberrant, and prematurely transform into astrocytes (Anton et al., 1997; Schmid et al., 2003), suggesting that neuregulin1 signaling normally suppresses astrocyte formation in vivo.

Evidence from a recent study further supports a role for neuregulin signaling in suppressing astrocyte formation. The embryonic lethality of erbB4 knockout mice can be rescued by reintroducing ErbB4 in in the heart (Tidcombe et al., 2003), thereby permitting the study of erbB4-deficient forebrain development. When compared to control littermates, the cortical levels of s100b and gfap mRNA are prematurely elevated in these mice at E17.5, a time point coinciding with the start of astrocyte formation (Sardi et al., 2006), suggesting that ErbB4 signaling controls the onset of astrogenesis in vivo by negatively regulating astrocyte formation. The same study provided a novel, kinase-signaling independent mechanism for this regulatory effect. They present data supporting a model whereby Nrg1-mediated activation of ErbB4 promotes receptor phosphorylation and its association with TAB2 and N-CoR. Ligand binding induces a presenilin-dependent cleavage of the
intracellular domain (ICD) of ErbB4, allowing for the translocation of the ErbB4ICD/TAB2/N-CoR complex to the nucleus, where it binds and transcriptionally represses the \textit{s100b} and \textit{gfap} promoters, and prevents their activation by factors that induce astrocyte differentiation. Consistently, transfection of a cleavage-sensitive or of the intracellular domain of ErbB4 (ErbB4ICD) but not cleavage resistant form of ErbB4 or kinase-dead ErbB4ICD into ErbB4-/- HER4 hearts cortices could significantly reduce the premature astrocyte formation in these embryonic lethality rescued ErbB4-/- mice, suggesting that canonical RTK signaling by ErbB4 is not sufficient and that the intracellular domain of ErbB4 is necessary and sufficient to regulate astrocyte formation in this context (Sardi et al., 2006). These studies support a role for neuregulin/ErbB signaling in promoting precursor self-renewal and inhibiting differentiation. N-CoR has previously been shown to complex with the Notch-effector RBP-J, and to bind and represses the \textit{gfap} promoter, an inhibition which is relieved by stimulation with CNTF, a gliogenic cytokine (Hermanson et al., 2002). All these studies support a model where coincident ErbB2/ErbB4 and Notch activation cause formation of an ErbB4ICD/TAB2/N-CoR/RBP-J complex that directly represses gliogenic genes, thereby controlling the timing of astrocyte formation. When precursors are exposed to increasing amounts of gliogenic cytokines (discussed in a later section), N-CoR translocates to the cytoplasm (Hermanson et al., 2002), STATs are activated, and if in a permissive cellular context, mediate gliogenic gene expression.

Neuregulins are also well known for their regulation of oligodendrocyte development. Inhibiting neuregulin1 signaling via ErbB3 receptors in striatal precursors cultured as neurospheres results in decreased proliferation and in increased apoptosis, leading to smaller neurospheres, and increased differentiation into oligodendrocytes (Calaora et al., 2001). Consistently, neuregulin signaling is also mitogenic for early oligodendrocyte precursors, but inhibits their terminal differentiation (Canoll et al., 1996).

a) iv. Neurotransmitters regulate neural precursor proliferation

A variety of neurotransmitters and their cognate receptors are also expressed in the VZ/SVZ during early embryonic forebrain development, suggesting that these play important roles prior to the establishment of cortical and subcortical synapses, and potentially regulate neural precursors. Small molecules such as glutamate and GABA, and AchR have been
implicated in neural precursor development and can all signal through different types of receptors. The first type is ionotropic receptors, which are ligand-gated ion channels that modulate calcium (Ca++) and sodium (Na+) ion influxes respectively. Ions such as Ca++ act as secondary messengers to activate various signaling cascades. The second type of receptors are seven-pass transmembrane G-protein coupled receptors (GPCR), where ligand binding activates various signaling cascades including PKC, PKA, and MAPK signaling (reviewed in Nguyen et al., 2001; Wang et al., 2007) and RhoGTPase-mediated signaling (Sah et al., 2000) via specific heterotrimeric G-protein activation. A variety of these types of receptors are expressed in the ventricular/subventricular zone during embryonic development (reviewed in Nguyen et al., 2001).

The role of neurotransmitter receptors in cortical precursor fate determination has mainly been studied in cultured precursors, whereas \textit{in vivo} data is surprisingly lacking in this field. Glutamate levels are high during cortical development and can influence neural precursor proliferation and neurogenesis by activating a variety of both ionotropic and metabotropic glutamate receptors (iGluR and mGluR) in a spatio-temporal specific manner (LoTurco et al., 1995; Haydar et al., 2000; Luk & Sadikot, 2004; Di Giorgi-Gerevini, 2005; Brazel et al., 2005; Schlett et al., 2006; Castiglione et al., 2008; Gandhi et al., 2008). \gamma-aminobutyric acid (GABA) is also abundant during mammalian brain development (Cicirata et al., 1991; Miranda-Contreras et al., 1998, 1999, 2000) and also regulates cell cycle kinetics in neural precursors and neuronal differentiation (LoTurco et al., 1995; Haydar et al., 2000; Antonopoulos et al., 1997). Lastly, activation of acetylcholine muscarinic receptors (GPCRs) also appears to influence proliferation and neurogenesis during cortical development by activating Ca++-dependent pathways as well as PI-3K and MAPK signaling (Ma et al., 2000; Li et al., 2001; Zhao et al., 2003; reviewed in Ma et al., 2004).

Although neurotransmitters, and other GPCR ligands (which will be discussed in section E below), have been implicated in the control of embryonic as well as adult neural precursor proliferation and differentiation, the precise populations of precursors that are subject to their regulation and the nature of the receptors and signaling pathways that mediate these functions are largely unknown, and represent a large area for potential future studies.
b) Extracellular signals regulating neurogenesis

Interestingly, while growth factors such as FGF and EGF signal through receptor tyrosine kinases to exert a mitogenic effect on neural precursors, the PDGF family of growth factors and neurotrophins NT-3 and BDNF which also signal through receptor tyrosine kinases and activate similar cascades promote neuronal differentiation. How different responses to these growth factors are regulated is currently unclear but most likely depends on timing, extent of signaling cascade activation, and cellular context.

One of our lab’s primary focus has been to define neurogenic growth factor-mediated signaling cascades that converge onto regulators of cell cycle exit and/or neurogenic transcription factors to regulate neurogenesis during cortical development. Studies from our lab and others support a role for a neurotrophin-activated Raf-MEK-ERK-C/EBP signaling cascade in the differentiation of neural precursors in culture and in vivo.

b) i. Neurotrophins regulate survival, proliferation and differentiation of precursors

The family of neurotrophic growth factors which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) is known to regulate many aspects of nervous system development (reviewed in Kaplan & Miller, 2000). Neurotrophins signal through Trk or p75 receptor tyrosine kinase receptors with differing affinities (reviewed in Dechant et al., 1994) to activate Ras-MEK-ERK as well as PI3-K signaling cascades (reviewed in Kaplan & Miller, 2000). In the context of forebrain development, ventricular zone precursors express NT-3 and BDNF (Maisonpierre et al., 1990; Fukumitsu et al., 1998; Barnabe-Heider et al., 2003) as well as their high-affinity receptors TrkB (Barnabe-Heider et al., 2003; Bartkowska et al., 2007) and TrkC (Tessarollo et al., 1993; Allendoerfer et al., 1994; Lamballe et al., 1994; Ghosh & Greenberg, 1995; Fukumitsu et al., 1998; Barnabe-Heider et al., 2003; Bartkowska et al., 2007) but do not express NGF and TrkA (Barnabe-Heider et al., 2003). The precise expression of these proteins in ventricular versus subventricular zone precursors is unclear, as is the expression of p75 neurotrophin receptor and truncated forms of Trk receptors. While the embryonic cortical ventricular/subventricular zone expression of p75 has not been shown, it is expressed in forebrain-derived neurosphere precursors (Lachyankar et al., 1997; Hosomi et al., 2003), adult SVZ neurogenic precursors (Young et al., 2007), suggesting that it may also regulate
important aspects of cortical precursor fate determination. The expression of truncated forms of TrkB and TrkC, produced by alternative splicing of Trk mRNAs and function by sequestering neurotrophins or acting as dominant negative forms of trks, is also unclear in the context of neural precursor development.

What is the role of endogenously-secreted neurotrophins during cortical development? Mice lacking NT-3, BDNF, TrkB or TrkC display CNS phenotypes including cortical abnormalities (Minichiello & Klein, 1996; Alcantara et al., 1997; Martinez et al., 1998; Ringstedt et al., 1998; Kahn et al., 1999; Xu et al., 2000; Lotto et al., 2001). However, these studies were not informative with regard to the precise role of the neurotrophins endogenously produced by cortical precursors. Interception of endogenous BDNF and NT-3-mediated Trk receptor activation in cultured precursors using function-blocking antibodies or pharmacological inhibitors revealed a requirement for endogenously-produced neurotrophins in precursor and neuronal survival (Barnabe-Heider et al., 2003), making it difficult to ascertain a role for neurotrophins in proliferation and differentiation. Consistently, inactivation of TrkB or TrkC or both by transfection of dominant-negative Trk expression vector or short-hairpin RNA vectors increases apoptosis in cultured precursors (Bartkowska et al., 2007), an effect which can be abolished by culturing the precursors in the presence of ZVAD, a pharmacological pan-caspase inhibitor. In this context, Trk inactivation results in decreased precursor proliferation (Bartkowska et al., 2007), suggesting that endogenously produced neurotrophins directly promote the proliferation of cortical precursors. Similarly, inactivation of Trk receptors by in utero electroporation of DN-Trk or TrkB/C shRNAs resulted in decreased proliferation of precursors followed by decreased neurogenesis, without affecting survival (Bartkowska et al., 2007), suggesting that other survival signals can compensate in vivo, but that neurotrophins are required for proliferation and neuronal differentiation.

Consistently, overexpression of BDNF using in utero electroporation of E13 precursors in vivo promotes proliferation and enhances neurogenesis (Bartkowska et al., 2007). Similarly the injection of NT-3 in the lateral ventricles of E13 embryos increases neurogenesis in a two-step manner, first by increasing proliferation of radial precursors and then increasing their neuronal production (Ohtsuka et al., 2008), suggesting that neurotrophins can regulate proliferation and differentiation of precursors in vivo. These
effects are reminiscent of the increase in precursor proliferation and increased neurogenesis in experiments where FGF2 was injected in the lateral ventricles of embryos (Vaccarino et al., 1999). Interestingly, NT-3 has been found to antagonize the proliferative effect of FGF2 on cultured embryonic cortical precursors (Ghosh & Greenberg, 1995). As mentioned in the previous section, the PI-3K and MAPK pathways are both required for FGF2-induced proliferation of cortical precursors (Learish et al., 2000; Jin et al., 2005; Shimizu et al., 2008), where the suppression of both pathways using pharmacological inhibitors reduces proliferation in an additive manner (Jin et al., 2005). PI-3K activation stimulates serine/threonine kinases such as Akt and/or ILK, which directly phosphorylate GSK3 to switch off its catalytic activity (Cross et al., 1995). GSK3α and GSK3β proteins are highly expressed in the developing brain (Woodgett, 1990), where they could play an important role in regulating proliferation, as in nonneural systems (Cui et al., 1998; Liang & Slingerland, 2003). Stimulation by EGF and FGF leads to GSK3 inactivation, resulting in the activation of pathways that are normally repressed by GSK3 (Cross et al., 1995; Shaw & Cohen, 1999). In a hippocampus-derived neurosphere system, it was demonstrated that NT-3 inhibits FGF-2-induced phosphorylation of Akt/GSK3β and precursor proliferation in a dose-dependent manner, without affecting ERK1/ERK2 phosphorylation (Jin et al., 2005), suggesting that NT-3 inhibits the FGF2-activated PI-3K/GSK3β pathway, which is required for precursor proliferation. Consistently, the inhibitory effect of NT-3 could be rescued by adding a pharmacological GSK3 inhibitor. Similarly, increasing akt levels in precursors results in increased survival, proliferation and self-renewal (Sinor & Lillien, 2004). The effects of increasing or inhibiting ILK activity in a similar context have not been published.

How does NT-3 inhibit the activation of PI-3K/Akt by FGF receptors? Interestingly, the activation of TrkC by NT-3 was shown to induce a protein-protein interaction at the level of phosphorylated TrkC and FGFR1 receptors (without affecting FGFR1 phosphorylation), and was suggested to be responsible for this antagonism, possibly by causing a conformational change in FGFR1 that could change its effectiveness in activating the PI-3K/Akt/GSK3β pathway (Jin et al., 2005). It is also possible that increased MAPK activation by NT-3 induces a negative feedback loop onto FGF2-mediated signaling, for example by inducing threonine phosphorylation of the FGFR stimulated 2α protein (FRS2α), a docking protein that interacts with FGF2 and other receptor tyrosine kinases. Threonine
phosphorylation of FRS2α causes reduced FRS2-mediated MAPK and PI-3K activation (Ong et al., 2001; Lax et al., 2002).

As described earlier, neurogenesis necessitates the coupling of cell cycle exit and neuronal differentiation. How neurotrophins and growth factors such as PDGF promote neurogenesis remains controversial. Some studies have shown that NT-3 exerts an anti-mitogenic effect on cultured precursors by upregulating p27kip2, a negative regulator of G1/S phase progression, and downregulating positive regulators such as cyclin D and cdks, causing a lengthening of G1 and promoting cell cycle exit (Lukaszewicz et al., 2002). However, several studies have also suggested that NT-3 is a differentiation factor that does not affect proliferation (Averbuch-Heller, 1994; Lachyankar et al., 1997; Hu et al., 2004). These discrepancies might be explained by differences in culture conditions and range of NT-3 doses used. For example, in the above study, NT-3 could inhibit FGF2 induced precursor proliferation at low doses (0.1-3 ng/mL), but not at the high doses (30-100ng/mL) which have been used in other studies (Jin et al., 2005). This suggests that NT-3 receptors with different affinities or that the degree of TrkC activation can modulate effects on proliferation and differentiation. These possibilities have not been studied, but it remains possible that neurotrophins coordinate both cell cycle exit and neuronal differentiation, two processes that are necessarily coupled during neurogenesis.

What are the important signaling cascades mediating neurotrophin function? Western blot analysis of cortical precursor culture lysates demonstrate that BDNF and NT-3 treatment activates the PI-3K and MEK/ERK signaling cascades, while inhibiting neurotrophin action using function blocking antibodies results in decreased activation of these signaling pathways (Barnabe-Heider et al, 2003), suggesting that PI-3K and MEK signaling regulate important functions of the neurotrophins. Inhibiting PI-3K by adding pharmacological inhibitors to cortical precursors that endogenously secrete NT-3 and BDNF had profound effects on cell survival, whereas inhibiting MEK activity had no effect on survival or proliferation of precursors cultured in the presence of FGF2 (Barnabe-Heider et al., 2003), suggesting that PI-3K regulates survival, and potentially proliferation, although this was not confirmed in the context of experiments where the primary cell survival defect was rescued. The importance of other signaling cascades such as PLC-γ/PKC (McCarty & Feinstein, 1999) which are also activated in response to trk activation were not investigated in these studies. However, the
PLC-γ/PKC signaling cascade which activates secondary messengers such as IP3 and intracellular Ca++ has been shown to regulate mitogenesis and fate in response to other RTK or GPCR-activating growth factors in neural precursors (Nicot et al. 2001; Lai & Feng, 2004).

MAPK signaling has been implicated in many aspect of neural precursor fate determination. In simple terms, MAPK signaling activation starts at autophosphorylation of dimerized receptor tyrosine kinases upon ligand binding, and recruitment of adaptor proteins which activate RasGTPase proteins. A cascade of kinases amplifies this signal, in a Ras-Raf-MEK-ERK order (Figure 1.4 describes the major proteins involved in this cascade). In the context of cortical development, Braf is required for precursor survival, proliferation, neuronal differentiation and migration (Camarero et al., 2006). In turn, inhibiting MEK activity using a dominant-negative form of MEK or pharmacological inhibitors results in decreased neurogenesis without any effects on survival. While inhibiting MEK activity does not affect proliferation in adherent precursor cultures (Ménard et al., 2002; Barnabé-Heider et al., 2003), others have shown that it is required in the context of neurosphere formation (Huang et al., 2008). Inhibiting MEK in vivo by in utero electroporation of a dominant-negative MEK in E14 precursors also reduces neurogenesis, and concomitantly increases the proportion of dividing precursors in this setting (Paquin et al., 2005), suggesting that MEK signaling is not required for proliferation in vivo at timepoints when neurogenesis has started, in agreement with previous studies showing that FGF2-induced proliferation is MAPK-dependent in early but not late precursors (Thomson, 2007). In agreement with these studies, acute inactivation of ERK5 in E13 cultured precursors decreases neurogenesis, and concomitantly increases proliferation (Liu et al., 2006). Conversely, increasing MEK or ERK activity by transfecting E13 cultured precursors with wild-type or constitutively active forms of MEK or constitutively active ERK5 can increase neurogenesis (Ménard et al., 2002; Liu et al., 2006), suggesting that MEK/ERK activity is required and sufficient for neurogenesis. On the other hand, in a phenotype closely resembling that of the fgf2-deficient precursors, conditional inactivation of erk2 in precursors of the developing forebrain results in a reduction in cortical thickness that is attributable to impaired proliferation of neural precursors during the neurogenic period, and to the generation of fewer neurons (Samuels et al., 2008), consistent with an early requirement of MEK-ERK signaling in regulating precursor proliferation as well as neuronal differentiation.
Ultimately, ERK activation mediates the relay of extracellular information to control transcriptional events in the nucleus, in a context-dependent manner. Acute versus sustained ERK activation can lead to differential proliferative and differentiative responses, respectively (D’Alessio et al., 2007). Multiple transcription factors are targets of ERK-mediated phosphorylation, including other kinases such as the 90kDa Ribosomal S6 kinases (Rsk) (reviewed in Anjum & Blenis, 2008). As discussed in a previous section, the neurogenic function of the transcription factor C/EBP is activated by ERK and Rsk-mediated phosphorylation (Ménard et al., 2002; Paquin et al., 2005). C/EBPs can then directly transactivate genes such as those encoding α1-α-tubulin and some neurogenic bHLHs, as discussed previously (Ménard et al., 2002; Uittenbogaard et al., 2007). Furthermore, BDNF and NT-3, for example, have been found to increase the expression of the neurogenic bHLHs such as Mash1 and Math1 at the mRNA level in cycling mouse neural precursors in culture, resulting in induction of NeuroD expression and increased neuronal differentiation following FGF2 withdrawal (Ito et al., 2003), suggesting that C/EBPs may also potentiate the expression of proneural bHLHs during neurogenesis. Altogether, these studies provide a model whereby neural precursors secrete their own neurotrophins to regulate the timing of neurogenesis by activating the MEK-ERK-Rsk-C/EBP pathway, leading to the transactivation of genes that promote neurogenesis and inhibit gliogenesis.

b) ii. PDGF signaling

PDGF ligands consist of –A,-B and/or –C chains, which can dimerize or heterodimerize and bind to the tyrosine kinase receptors PDGFRα and β with different affinities (reviewed in Tallquist & Kaslauskas, 2004). With regards to CNS development, PDGF-A,-B and PDGFRβ knock-out mice only display minor CNS defects (reviewed in Betsholtz et al., 2004), where, for example, PDGF-A deficient mice show defects in oligodendrocyte precursor development (Fruttiger et al., 1999). PDGFRα-deficient or mutated mice die in utero, and display severe neural tube and neural crest defects, including collapsed ventricles (Soriano et al., 1997; Stephenson et al., 1991), suggesting that PDGFRα signaling is required for CNS development, and that compensatory effects may be masking the requirement of PDGF signaling during cortical precursor development.
PDGFRα transcripts are detected as early as E8.5 in neuroepithelial ventricular zone precursors, and are also expressed in early-born neurons (Andrae et al., 2001), as well as glial precursors (Hart et al., 1989; Pringle et al., 1992; Yeh et al., 1993), suggesting that PDGFRα signaling regulates many aspects of nervous system development. Similarly, PDGFRα as well as PDGFRβ are expressed at the protein level in cultured neuroepithelial precursors (Williams et al., 1997; Park et al., 1999). Interestingly, PDGFRα expression is restricted to apical end feet in embryonic ventricular zone precursors (Andrae et al., 2001), suggesting that this may be yet another example of receptor that segregates equally or unequally during developmental precursor mitoses. Further support for this comes from a study of adult neural stem cells where PDGFRα, along with EGFR, are found in apical cilium of quiescent ependymal cells or cycling neuroblasts (Danilov et al., 2008) but translocate to mitotic spindle microtubules throughout mitosis prior to their segregation to daughter cells (Danilov et al., 2008). While PDGF ligands are not expressed by cortical precursors (Williams et al., 1997), ventricular zone PDGFRs are activated in vivo during early embryonic development (Williams et al., 1997), suggesting that they are expressed by other cell types such as preplate neurons (Sasahara et al., 1992). Consistently, adding exogenous PDGF ligands to cultured precursors promotes their differentiation into neurons (Williams et al., 1997; Park et al., 1999; Ménard et al., 2002) and inhibits astrocyte formation (Park et al., 1999), a process that requires de novo gene transcription (Williams et al., 1997; Park et al., 1999).

Altogether, this suggests that at least two families of growth factors, the neurotrophins and PDGF superfamily, regulate neuronal gene expression and control the timing of neurogenesis during cortical development.

**j) Extracellular signals regulating gliogenesis**

While growth-factor-mediated activation of a MEK-ERK-C/EBP signaling cascades promotes neurogenesis, multiple extrinsic signals, including EGFR (discussed above), BMPs (discussed in more detail below), and Notch (discussed above) promote astrocyte formation by activating or potentiating IL-6 cytokine family-driven JAK-STAT signaling.

**c) i. A central LIFRβ/gp130-JAK-STAT pathway drives astrocyte formation**
Cytokines of the IL-6 family which signal through a LIFR/gp130 homo- or heterodimers have been implicated in the instructive generation of astrocytes (reviewed in Catteneo et al., 1999). Cytokines of the IL-6 family that play a role during brain development include LIF, ciliary neuronotrophic factor (CNTF), cardiotrophin 1 (CT-1), and oncostatin M (OsM) (reviewed in Heinrich et al., 1998; Turnley & Bartlett, 2000), as well as neuropoietin & cardiotrophin-like cytokine (CLC) which have recently been identified in the developing brain (Derouet et al., 2004; Elson et al., 2000). These cytokines bind to transmembrane LIFRβ/gp130 dimers, with a few distinctions. For example, LIF can also bind to a LIFRβ homodimer with low affinity and CNTF binds a tripartite receptor composed of LIFRβ/gp130 and a membrane-linked CNTFRα component. (reviewed in Heinrich et al., 1998; Turnley & Bartlett, 2000). The cytoplasmic domains of LIFRβ and gp130 are associated with Janus kinase kinases (JAKs), a non-receptor tyrosine kinase, in an inactive state. In a cellular context-specific manner, ligand binding activates the JAKs which in turn phosphorylate several tyrosine sites in the cytoplasmic tail of the receptors that become phosphotyrosine docking sites for a variety of SH2 domain-containing cytoplasmic proteins. In the primary cytokine signaling JAK-STAT pathway, signal transducers and activators of transcription (STAT) proteins are recruited to specific LIFR/gp130 phosphotyrosine sites, and are in turn phosphorylated and activated by the JAKs, which mediates their dimerization and translocation to the nucleus where they regulate gene expression (as discussed in a previous section). STAT1 and STAT3 are the relevant mediators of IL-6-type cytokines signaling (Briscoe et al., 1994). Other SH2 domain-containing proteins such as src and SHP-2 can also associate with specific phosphotyrosine sites on the receptors, leading to activation of the Ras-mitogen-activated protein (MAP) kinase pathway, as well as the PI-3K pathway in some cell types (reviewed in Heirich et al., 1998; Ernst & Jenkins, 2004). Furthermore, certain phosphotyrosine residues are associated with the negative regulation of cytokine signaling, namely be recruitment of SOCS proteins (suppressor of cytokine signaling) and SHP-2, which can both downregulate gp130/JAK-STAT signaling (reviewed in Ernst & Jenkins, 2004).

In the context of cortical precursors, gp130/LIFRβ-JAK-STAT signaling is required for astrocyte formation. Lifrb-/- or gp130-/- mice show significant if not complete reduction in GFAP-immunoreactive astrocytes in vivo, and in cultured cortical precursors (Ware et al., 2004).
1995; Koblar et al., 1998; Nakashima et al., 1999b). Furthermore, acute knockdown of gp130 in precursors decreased the percentage of precursors that generate S100β, CD44 or GFAP+ve astrocytes in culture and in vivo (Barnabe-Heider et al., 2005), indicating that gp130 is required in a cell-autonomous manner for the generation of astrocytes. These studies also suggest that cytokines that signal through LIFRβ/gp130 receptors regulate astrocyte formation during brain development.

What is the relevant gliogenic cytokine during cortical development? Multiple studies have shown that LIF, CNTF, CLC and CT-1 are sufficient to induce astrocyte formation and inhibit neurogenesis in cultured cortical precursors, and that these actions require LIFRβ, gp130, JAK and STAT3 activation (Johe et al., 1996; Bonni et al., 1997; Rajan & McKay, 1998; Nakashima et al., 1999a, 1999b; Ochiai et al., 2001; Uemura et al., 2002; Barnabé-Heider et al., 2005). As discussed earlier, STAT activation is required for the direct transactivation of at least two astrocytic genes, glfap and s100b, via STAT binding sites within their promoters (Bonni et al., 1997; Nakashima et al., 1999a; Namihira et al., 2004). Furthermore, ectopic expression of CNTF in the ventricular zone during the period of neurogenesis induces premature astrocyte formation in vivo by the same mechanism (Barnabé-Heider et al., 2005), suggesting that at least a subset of precursors are competent to generate astrocytes during this period, and further suggesting that exposure to gliogenic cytokines can regulate the timing of astrogenesis. Although exogenous LIF and CNTF can potently induce astrocyte formation in culture and in vivo, these are unlikely to be the relevant gliogenic cytokines since neither of them are expressed at the onset of gliogenesis (Stockli et al., 1991; Patterson & Fann, 1992). Furthermore, lif-/- mice exhibit deficits in astrocyte numbers in the hippocampus and dentate gyrus but other brain regions are only modestly affected (Koblar et al., 1998; Bugga et al., 1998), while cntf-/- mice show no deficits in astrocyte formation (Masu et al., 1993). The finding that conditioned media derived from pure neuron cultures could promote premature astrocyte formation in cortical precursor cultures suggested that neurons might produce the relevant gliogenic cytokine(s) and regulate the onset of astrocyte formation in a molecular timer feedback mechanism. CT-1 protein expression was found to be restricted to newly-born neurons during cortical development, and ablation of ct-1 in vivo resulted in major deficits in cortical astrogenesis (Barnabe-Heider et al., 2005), suggesting that neuron-derived CT-1 regulates the onset of
gliogenesis \textit{in vivo}. Altogether, these studies support a model where the switch from neurogenesis to gliogenesis involves a feedback-loop, where neurons, the first born cell types, produce accumulating levels of CT-1 that instruct remaining precursors to stop making neurons and start generating astrocytes (Illustrated in Figure 1.1).

As mentioned above, cytokine signaling can also induce activation of MAPK and PI-3K in a cell context-specific manner. The role of cytokine-induced MAPK signaling in the context of cortical precursor biology is not clear, with different studies giving contradicting results (Bonni et al., 1997; Rajan & McKay, 1998; Ménard et al., 2002).

b) ii. BMPs are multifunctional during cortical development, and are associated with the regulation of gliogenesis

Bone morphogenetic protein (BMP) signaling appears to cooperate with many extracellular factors during embryonic development, including cytokine-induced JAK-STAT signaling. BMPs are cytokines that belong to the transforming growth factor-\(\beta\) (TGF-\(\beta\)) superfamily and have pleiotropic effects during development depending on timing of activation (reviewed in Hall & Miller, 2004). BMP2, BMP4 and BMP7 are the relevant BMPs that regulate cortical precursors. Their expression in radial precursors and endothelial cells is regulated by Wnt signaling and epigenetic mechanisms (Kasai et al., 2005; Shaked et al., 2008; Imura et al., 2008). The actions of BMPs are mediated by heterodimerization of type I and type II ligand binding and serine/threonine kinase receptors. Receptor activation leads to recruitment and phosphorylation of the downstream transcription factors Smad1,-5, or -8 at serine residues, inducing the formation of a complex with Smad4, a common mediator of BMP signaling (reviewed in Chen et al., 2004).

The effect of exogenous BMP signaling on cortical precursors varies during cortical development, where BMPs can promote proliferation, neurogenesis, and gliogenesis in early and late precursors respectively (Mehler et al., 2000). Smad1 can interact with many transcriptional regulators, often linking transcription factors and the p300 co-activator to regulate transcriptional activation during precursor maintenance (Lillien & Raphael, 2000; Takizawa et al., 2003), neurogenesis (Sun et al., 2001), astrocyte formation (Nakashima et al., 1999a; Rajan et al., 2003; Fukuda et al., 2007), and adult neurogenesis (Colak et al., 2008). Thus, by interacting with and potentiating the actions of neurogenic and gliogenic
transcription factors, BMP signaling dually promotes neurogenesis during the neurogenic period (Li et al., 1998; Mabie et al., 1999) and astrocyte formation during the gliogenic period (Gross et al., 1996; Gomes et al., 2003).

Another interesting interaction exists between JAK-STAT signaling and BMP signaling, where LIF-mediated STAT3 activation induces BMP2 expression in precursors, which in turn potentiates STAT3 transcriptional activity by activating Smad1 in a positive feedback loop that promotes astrocyte formation (Fukuda et al., 2007). Furthermore, in collaboration with Notch signaling, BMPs can also promote astrocyte formation and suppress neurogenesis, at least during the period of gliogenesis, by inducing the expression of Notch effector Hes5 (Takizawa et al., 2003) and dominant-negative bHLHs Id1 and Id3, which repress neurogenesis and oligodendrocyte formation (Nakashima et al., 2001).

While BMPs appear to be multifunctional, the requirement for BMP signaling in regulating cortical precursor maintenance, neurogenesis and gliogenesis is still unclear and remains a big issue. What are the consequences of inhibiting BMP signaling? BMP receptors have been inactivated in the developing mouse neural tube using Foxg1 promoter-driven cre expression in a bmpr1a<sup>en</sup> and bmpr1b<sup>-/-</sup> background (Fernandes et al., 2007). However, these mice die around E11.5, prohibiting the analysis of precursor fate determination. Furthermore, although floxed BMP2 (Tsuji et al., 2006) and BMP4 (Bandyopadhyray et al., 2006) mice exist, CNS-specific phenotypes have not yet been published. Inhibiting BMP signaling in cultured cortical precursors by treating them with noggin, a BMP antagonist, results in decreased astrocyte formation and increased oligodendrocyte formation (Kasai et al., 2005; Mehler et al., 2000), suggesting that BMPs normally promote astrocytic and suppress oligodendrocytic fates. BMPs could be doing so by upregulating the dominant negative bHLHs Id1 and Id3, as mentioned above, which can antagonize olig1 and olig2, pro-oligodendrogenic bHLHs. However, more loss-of-function experiments are required to determine the requirement of BMP signaling in cell fate determination.

In summary for this section, while many signaling pathway such as Notch, BMPs and EGFR promote astrocyte formation, they cooperate with the central LIFRβ/gp130-JAK-STAT cascade which is required for precursors to become astrocytes.
**Rationale for 1st data chapter:**

Implicit to any model where growth factors influence neural cell fate is the assumption that multipotent precursors are able to respond to both neurogenic and gliogenic factors even at timepoints when those cell types are not normally generated. The majority of cortical precursors express neurogenic and gliogenic receptors coincidentally (Parks et al., 1999) and are exposed to conflicting signals at certain developmental timepoints. While many intrinsic mechanisms render cortical precursors increasingly responsive to gliogenic cytokines as development proceeds (discussed above and reviewed in Miller and Gauthier, 2007), it is nonetheless clear that many cortical precursors are capable of making astrocytes even during the neurogenic period if exposed to gliogenic cytokines (Morrow et al., 2001; Barnabé-Heider et al., 2005). Mechanisms must therefore exist to ensure that precursors enact only one cell fate decision even when exposed to multiple, contradictory growth factor cues. Previous work indicates that such biasing mechanisms do exist in neural precursors since growth factors that promote gliogenesis decrease neurogenesis (Shah et al., 1996; Bonni et al., 1997; Yanagisawah et al., 2001; Barnabé-Heider, 2005), and the neurogenic growth factor PDGF can, in some conditions, “override” the gliogenic effect of CNTF in cultures of cortical precursors (Park et al., 1999). In the first part of my thesis, I have asked about potential growth factor-regulated signaling proteins that might mediate these effects by promoting one neural cell fate while at the same time inhibiting another, and have identified SHP-2.

**C) SHP-2 and its potential role in integrating neurogenic and gliogenic signals during cerebral cortex development**

SHP-2 is a growth factor-regulated protein tyrosine phosphatase that is expressed in most tissues, including the central nervous system, where it is expressed in the ventricular zone precursors as well as cells in the cortical mantle during embryonic development (Reeves et al., 1996; Servidei et al., 1998). SHP-2 is part of the Src homology protein tyrosine phosphatase (SHPTP or SHP) family, with also includes SHP-1 (reviewed in Neel et al., 2003; Dance et al., 2008). The protein structure of SHP-2 consists of two src homology domains (N-SH2 and C-SH2) as the amino-terminus, a single PTP (phosphatase) region
which mediates binding and dephosphorylation of substrates, as well as a c-terminal hydrophilic tail containing consensus tyrosine phosphorylation sites, which can mediate interactions with other proteins. Under basal conditions, SHP-2 is kept catalytically inactive by an intramolecular inhibitory interaction between its N-SH2 and PTP domains. Via its SH2 domains, SHP-2 is recruited either directly to consensus phosphotyrosine sites on the intracellular domains of many receptor tyrosine kinases, cytokine receptors, and integrin receptors upon their activation, or indirectly by interacting with a variety of adaptors, including FRS2α, Gab and IRS proteins, that bind these receptors. This releases the autoinhibitory interactions, resulting in the catalytic activation of the “open” SHP-2 conformation. How the phosphorylation of tyrosine sites at the SHP-2 c-terminus is mediated or regulated is not clear, as is the importance of these regulatory sites. However, independent data also argue that SHP-2 tyrosine phosphorylation mediates its role as an adaptor protein (reviewed in Dance et al., 2008) or potentiates phosphatase activity in response to some but not all growth factors (Araki et al., 2003).

Upon its activation, SHP-2 mediates the transmission of intracellular signaling pathways including Ras-MEK-ERK and PI-3K (Zhang et al., 2002; D’Alessio et al., 2003). In many cell types cells, SHP-2 activity is required for sustained receptor tyrosine kinase-mediated Ras-MEK-ERK activation (reviewed in Neel et al., 2003; Dance et al., 2008). This is also true in the nervous system, where, for example, SHP-2 is a positive regulator of TrkB-mediated ERK activation in cortical neurons (Easton et al., 2006). The mechanisms involved in this are still, if not elusive, a matter of debate in the research community. It is thought that SHP-2 mediates this positive regulation primarily by dephosphorylating and inactivating proteins that negatively regulate Ras signaling (Neel et al., 2003; Dance et al., 2008).

SHP-2 is also recruited to specific phosphotyrosine sites on the LIFRβ and gp130 co-receptors, leading to its activation in the context of cytokine signaling as well. In this context, SHP-2 can induce Ras-MEK-ERK activation, but has also been associated with negative regulation of gp130-mediated JAK-STAT signaling (Ohtani et al., 2000; Bartoe & Nathanson, 2002; Lehmann et al., 2003; Ernst and Jenkins, 2004; Clahsen et al., 2005), which as discussed above instructively directs astrogenesis (Bonni et al., 1997; Rajan & Mckay, 1998; Barnabé-Heider, 2005), and may also regulate precursor self-renewal (Yoshimatsu et al., 2006). We have proposed that since SHP-2 functions as a positive regulator of growth
factor-mediated MEK-ERK activation and as an inhibitor of JAK-STAT signaling, it is an ideal candidate to act as a signaling switch in promoting neurogenesis and at the same time inhibiting gliogenesis.

In non-neural cell types, SHP-2 is required for many cellular processes including survival, proliferation, differentiation and migration. Full SHP-2 knockout mice die early in gestation due to gastrulation defects (Saxton et al., 1997), illustrating the importance of SHP-2 during embryonic development. Although SHP-2 is known as an important regulator of extrinsic cue-mediated signaling cascades that, as discussed in previous sections, are required for neural precursor proliferation, neurogenesis and gliogenesis, the role of SHP-2 in the developing central nervous system has largely been unexplored. The requirement for SHP-2 in neural development was addressed by one previous study, using a transgenic catalytically inactive form of SHP-2 expressed under the control of the nestin promoter (Aoki et al., 2000). Although the SHP-2 transgene was expressed in ventricular zone precursors, this group found no abnormalities with regard to precursor proliferation and migration. In an ischemic injury model however, transgenic SHP-2 neurons showed increased susceptibility to neuronal death (Aoki et al., 2000). There were integral issues with this study. First, as the authors themselves noted, the level of SHP-2 expression was inconsistent. Furthermore, the dominant-negative SHP-2 mutant used in this study was a point mutant, which has no kinase activity but can still bind substrates, thereby acting as a substrate-trapping mutant. As discussed above, the majority of known SHP-2 substrates are negative regulators of Ras activation, and their sequestration by a kinase-dead SHP-2 would also result in their inactivation, mimicking normal SHP-2 activity. Therefore, it is my opinion that this study did not convincingly rule out a major role for SHP-2 in central nervous system development.

Further support for the idea that SHP-2 may regulate neural development comes from the genetically-defined human Noonan Syndrome, where mutations rendering SHP-2 active or more readily activatable result in many developmental defects, including perturbed cortical development and mental retardation.
D) Neuro-cardio-facio-cutaneous syndromes

Noonan syndrome (NS) is part of a family of related disorders that were recently classified as Neuro-Cardio-Facial-Cutaneous syndromes, and which also include the severe cardio-facial-cutaneous (CFC), Costello, Neurofibromatosis (NF), and LEOPARD disorders (reviewed in Bentires-Alj et al., 2006; Denayer et al., 2007). These congenital disorders share an overlapping spectrum of phenotypes including cardiac defects, craniofacial abnormalities, growth retardation as well as mild to severe mental retardation, making it historically difficult to diagnose them accurately. Within the last five years, breakthroughs in the genetic screening of these conditions have allowed to clinically classify these disorders on a genetic as well as physiological basis. Interestingly, all of the mutations identified thus far are in different genes that encode modulators or effectors of the Ras-MEK-ERK pathway, most of them being activating or gain-of-function mutations (most recently reviewed in Aoki et al., 2008). (Illustrated in Figure 1.5) In about fifty percent of cases, Noonan Syndrome is caused by missense mutations in the human \textit{ptpn11} (SHP-2) gene, often resulting in expression of a SHP-2 protein with increased basal or stimulated phosphatase activity (Tartaglia et al., 2001). All mutations identified so far are in the N-terminal SH2 domain or in the phosphatase (PTP) domain, disrupting the autoinhibitory interaction that regulates SHP-2 activity, and creating an open SHP-2 conformation with increased SHP-2 activity under basal conditions (Keilhack et al., 2005; Niihori et al., 2005; Bocchinfuso et al., 2007). Mutations in the gene encoding SOS, a Ras-GEF, are the second most frequent, occurring in about 20% of NS patients, (Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007), while 2-5% are associated with Kras (Carta et al., 2006; Schubbert et al., 2006; Zenker et al., 2007), and c-Raf (Raf1) mutations have recently been identified in a few patients (Pandit et al., 2007; Razzaque et al., 2007). LEOPARD syndrome is also caused by different mutations in the gene encoding SHP-2 (Digilio et al., 2002; Legius et al., 2002). Unlike Noonan-associated SHP-2 mutations however, biochemical analysis suggest that LEOPARD-associated SHP-2 mutants show decreased catalytic activity, and act as dominant-negative forms of SHP-2 (Tartaglia et al., 2006; Kontaridis et al., 2006). However, given that the phenotype of Noonan and LEOPARD syndromes overlap significantly, it has been proposed that the LEOPARD-associated SHP-2 mutations have gain-of-function effects, perhaps by
altering the conformation of SHP-2 and altering its substrate interactions (reviewed in Edouard et al., 2007; Oishi et al., 2008). With regards to the other NCFCs, over 90% of Costello cases are caused by gain-of-function H-Ras mutations (Aoki et al., 2005; Estep et al., 2006; Gripp et al., 2006). The majority (40-75%) of cardio-facial-cutaneous (CFC) disorders are caused by Braf mutations (Niihori et al., 2006; Rodrigues-Viciana et al., 2006), while 5-10% of cases are caused by mutations in MEK1 or MEK2 (Rodrigues-Viciana et al., 2006; Narumi et al., 2007; Schulz et al., 2008) or Kras (Schubbert et al., 2006; Niihori et al., 2006).

In Noonan Syndrome, children present with cardiac defects, craniofacial abnormalities, short stature (Noonan, 1994), increased propensity to develop juvenile myelomonocytic leukemia (JMML) (Kratz et al., 2005) and a large percentage (1/3 to 1/2) exhibit learning disabilities and mild mental retardation (Noonan, 1994; Yoshida et al., 2004; Lee et al., 2005). While this genetic syndrome can be modeled in the mouse by knocking-in a Noonan Syndrome SHP-2 allele (Araki et al., 2004), it was not yet known whether this mouse displays neural perturbations that might explain the human cognitive deficits.

In the first part data chapter of this thesis, I explore the role of SHP-2 in regulating neurogenesis and gliogenesis, as well as the potential consequences of misregulating SHP-2 activity during cortical development. I hypothesize that SHP-2 promotes neurogenesis and inhibits gliogenesis to ensure that neural precursors only generate neurons during the neurogenic period, and further suggest that perturbations in cell genesis could contribute to the neurological dysfunction in Noonan Syndrome.

E) Lfc and its potential role in regulating spindle orientation and fate determination in radial precursors

In the second part of this thesis, we investigated the role of the Rho-guanine exchange factor (GEF) Lfc in the regulation of neural precursor divisions and fate determination. In this regard, Lfc has been shown to regulate mitotic spindle orientation in nonneural cells (Benais-Pont et al., 2003; Bakal et al., 2005), making it an interesting candidate to investigate within the context of symmetric versus asymmetric divisions that regulate self-renewal and neurogenesis during cortical development.

Lfc and its human orthologue, GEF-H1, are Dbl-family GEFs that have specific activity towards Rho (Glaven et al., 1996; Ren et al., 1998; Glaven et al., 1999; reviewed in
Rossman et al., 2005). Rho family GTPases which includes Rho, Rac and cdc42, play essential roles in regulating the dynamic rearrangement of actin and microtubule cytoskeletal structures in all cell types (reviewed in Heasman et al., 2008), and are thus critical players in many aspects of neural development. The DbI-like guanine nucleotide exchange factors (GEFs) serve as direct activators of these GTPases, by catalyzing the loading of GTP onto RhoGTPases. The Rho-GEF Lfc was first identified as a c-terminally truncated protein with oncogenic activity (Whitehead et al., 1995). Lfc, Lsc and Lbc are part of a subgroup of Dbl–related RhoGEFs and, unlike other RhoGEFs such as Dbl, Ost, and Dbs, appear to function as highly specific GEFs towards Rho. Neither Lfc nor Lsc show any ability to stimulate GDP dissociation from other related GTP binding proteins such as Rac, cdc42 or Ras (Glaven et al, 1996; Ryan et al., 2005). Importantly, Lfc shows a unique ability among GEFs in its (most likely indirect) association with microtubules, an interaction which is thought to suppress its activity (Krendel et al., 2002; Ryan et al., 2005). The regulatory and functional domains of Lfc are illustrated in Figure 1.6.

Microtubule-bound Lfc may thus be activated in response to stimuli that promote microtubule depolymerization or that release Lfc from direct or indirect inhibitory interactions at microtubules, leading to Rho activation at adjacent sites of actin reorganization to regulate cytoskeletal dynamics. Such a model is supported by two studies investigating the regulation and function of Lfc in two different systems. The first study demonstrates that microtubule-bound Lfc relocalizes to sites of actin-based stress fiber formation in response to LPA, a GPCR ligand. In this study, Lfc was required for LPA-mediated stress fiber formation in a Rho-dependent manner, and was activated via heterotrimeric Gα proteins (Greeve et al., submitted a.). Lfc is highly expressed in the developing brain, including in ventricular zone precursors and newly-born neurons (Yoshizawa et al., 2003; Ryan et al., 2005), where a similar mechanism regulates dendritic spine dynamics. Under basal conditions in neurons, exogenous Lfc was largely found in association with microtubules in the dendritic shafts. In response to activation by KCl depolarization or glutamate/NMDA stimulation, Lfc translocated to adjacent F-actin rich dendritic spines, via a direct interaction with actin-binding proteins and homologous protein phosphatase 1 (PP1) neurabin or spinophilin, to regulate dendritic spine morphology in a Rho-dependent manner (Ryan et al, 2005; Nakayama et al., 2000), thereby contributing to the
regulation of synaptic plasticity. In that study, increasing Lfc expression stabilized F-actin and decreased dendritic spine size (Ryan et al., 2005). Altogether, these studies suggest that extracellular signals mediate the translocation of microtubule-bound Lfc to actin rich sites to activate Rho and regulate cytoskeletal rearrangement linked to many fundamental cellular processes.

Lfc is also expressed in ventricular zone precursors throughout the period of neurogenesis (Yoshizawa et al., 2003). Although Lfc itself has not been previously studied in precursors, Rho family GTPases have previously been shown to play important roles as regulators of cell fate during neural development. More specifically, RhoA and cdc42a expression is restricted to ventricular zone precursors during prenatal mouse development, whereas RhoB and cdc42b are only expressed in the cortical plate where differentiated neurons settle, suggesting isoform-specific functions during neurogenesis and differentiation (Olenik et al., 1999). Cdc42, which is not activated by Lfc and related members, plays an important role in radial precursor fate determination by regulating the apico-basal polarity which is, as discussed in the first part of this chapter, essential for their maintenance as radial precursors (Cappello et al., 2006; Chen et al., 2006). In chick neuroepithelium, RhoA regulates mitotic spindle positioning and orientation during precursor mitosis, thereby regulating planar (symmetric) versus apico-basal (asymmetric) divisions in precursors without, however, affecting neurogenesis (Roszko et al., 2006). Little is known about the molecular mechanisms that regulate Rho at specific subcellular locations, but this most likely involves the precise temporal and spatial regulation of RhoGEFs and RhoGAPs. Immunofluorescence studies have revealed that Lfc colocalizes with mitotic spindle microtubules in non-neural cell types (Benais-Pont et al., 2003; Bakal et al., 2005), suggesting that it may play a direct role in activating Rho at specific sites of microtubule depolarization and actin remodeling, for example at sites where spindle poles are tethered to the cell cortex during the establishment or rotation of mitotic spindle orientation. In this regard, Lfc was found to promote spindle assembly in early mitosis of non-neural cell types and to regulate mitotic spindle orientation (Bakal et al, 2005), suggesting that it could regulate symmetric and asymmetric divisions during cortical development.
F) Tctex-1 and its potential role as a negative regulator of Lfc in regulation of fate determination

Interestingly, Lfc interacts with, and is negatively regulated by Tctex-1 (Greeve et al., submitted a), a protein that is expressed in adult neural stem cells (Dedesma et al., 2006). While most extensively characterized as a light chain component of the dynein motor complex responsible for cargo binding (King et al., 1996), Tctex-1 also has several dynein-independent functions as a nonreceptor activator of heterotrimeric G-protein signaling (Tctex-1 is also known as AGS2; Takesono et al., 1999; reviewed in Lanier, 2004). In a function requiring its interaction with Gβγ, Tctex-1 regulates RhoGTPase-dependent neuronal growth (Chuang et al., 2005; Sachdev et al., 2007). In these studies, overexpression of Tctex-1 increased neurite extension (Sachdev et al., 2007) while knocking down Tctex-1 inhibited neuronal growth (Chuang et al., 2005), mimicking RhoA inactivation or activation respectively. Although the involvement of Lfc was not addressed in these studies, it is interesting to speculate that it, or other Rho-GEFs, could be negatively regulated by Tctex-1 in this setting. In this regard, a recent study demonstrates that Tctex-1 inhibits Lfc-mediated actin stress fiber formation in response to G-protein activation induced by LPA, in a dynein-independent manner (Greeve et al., submitted a). As depicted in Figure 1.6, a region adjacent to the microtubule-binding domain in the N-terminal portion of Lfc is required for its interaction with Tctex-1 (Greeve et al., submitted a), and is also regulated by phosphorylation (Greeve et al., submitted b). Altogether, evidence suggests that Tctex-1 and Lfc may be part of a genetic pathway that coordinates microtubule and Rho-dependent actin cytoskeletal rearrangement at defined subcellular locations.

On that note, correct positioning and morphological changes of the mitotic spindle are cellular events that require the regulated interaction of microtubules and cortical actin, to which the centrosomal spindle poles are linked. As discussed earlier, a few centrosomal proteins have been shown to regulate mitotic spindle orientation and cell fate outcomes in dividing neural precursors (Feng et al., 2004; Tsai et al., 2005; Shu et al., 2006; Yingling et al., 2008; Pawlisz et al., 2008). Could Tctex-1 and Lfc function in a pathway that also participates in mitotic spindle regulation and fate determination?

Intriguingly, a recent study showed that AGS3, another nonreceptor activator of G-protein signaling (and a mammalian Pins homologue) and Gβγ signaling regulate mitotic
spindle orientation and neurogenesis in cortical precursors (Sanada and Tsai, 2005). Like Lfc, Tctex-1 also localizes to mitotic spindles in nonneural (Tai et al., 1998), and has been implicated in dynein-dependent transport of polarized molecules in epithelial cells (Tai et al., 1999), raising the possibility that Tctex-1 might segregate fate determinants in neural precursor cells (Dedesma et al., 2006).

In the second data chapter of this thesis, I have therefore tested the idea that Lfc and Tctex-1 proteins might be important in embryonic neural precursors, and demonstrate that Lfc and Tctex-1 are part of an essential pathway that regulates the genesis of neurons from radial precursor cells, potentially by regulating mitotic spindle orientation.
Figure 1.1 *Timing of cell genesis during cortical development.* Multipotent and more restricted precursors proliferate in the ventricular and subventricular zone, and give rise to neurons, astrocytes (and oligodendrocytes) in a sequential developmental timecourse. Neurogenesis takes place between E12 and E18, while astrocytes are born perinatally. Part of this timer is regulated by a feedback mechanism where newly-born neuron-derived production of cardiotrophin-1 instructs gliogenic precursors to generate astrocytes.
a) Neurogenic precursor

b) Gliogenic precursor

Figure 1.2
Figure 1.2: *Multiple extracellular signals and intrinsic programs regulate the neurogenic versus gliogenic potential of neural precursors.*

*a) Illustration of some of the most important extracellular and intrinsic mechanisms that work in concert to promote neurogenesis and repress gliogenesis during the neurogenic period.*

bHLH proteins including neurogenins (Ngn) and Mash1 directly promote the transcription of neurogenic genes and at the same time inhibit gliogenesis by sequestering the p300/CBP and BMP-activated Smad co-factors. Receptor tyrosine kinase receptors bind to growth factors such as the neurotrophins and PDGF, and directly activate a MEK-ERK-Rsk pathway that induces phosphorylation of the C/EBP family of transcription factors that, in turn, directly bind to and activate the promoters of neuronal genes such as α1 tubulin, and by unknown mechanisms also represses gliogenesis. Concomitantly, glial genes such as *gfap* and *s100b* are repressed via DNA methylation and MeCP2 binding which promotes the formation of an inactive chromatin conformation. Another repressive mechanism involves activation of the ErbB4 receptor by neuregulin, which causes translocation of NCoR repressor protein to the nucleus, where it complexes with the Notch effector RBP-J at the *gfap* promoter. At this stage, because of the combination of all these and other mechanisms, gliogenic gp130-JAK-STAT pathway activation is minimal, and is not sufficient to instruct gliogenesis.

*b) Multiple environmental cues converge to promote the gliogenic switch.* At least four different signals, FGF/EGF (not shown), CT-1, BMPs and Notch ligands, converge to regulate the appropriate timing of gliogenesis. FGFR and EGFR signaling have set the stage for gliogenesis, by relieving the epigenetic repression at gliogenic promoters. The neuronally-derived CT-1 cytokine binds to and activates the gp130 and LIFRβ coreceptors, which then signal via the JAKs to phosphorylate and activate the STAT1/STAT3 transcription factors. In a complex with the Smads and p300/CBP co-factors, the STATs bind and transactivate the promoters of gliogenic genes such as *gfap* and *s100b*, as well as components of the gp130-JAK-STAT pathway, which generates a positive feedback loop for the gliogenic pathway. They likely to so in combination with other transcription factors such as NFIs, and RBP-J Notch-effector, which is relieve from N-CoR repression in response to CT-1. BMP and Notch signaling also contribute to the repression of neurogenesis, by inducing Hes and Id transcriptional repressors, which inhibit the neurogenic bHLHs.
Mechanisms regulating neural precursor polarity and symmetric vs asymmetric divisions. Apically polarized fate determinants (shown as a red crescent) are segregated equally or unequally during symmetric and asymmetric divisions, generating daughter cells with equal or unequal fates, respectively. One mechanism that can account for a switch in symmetric to asymmetric divisions at the onset of neurogenesis is changes in mitotic spindle apparatus orientation. Polarity proteins (shown in red) including the par complex (mPar3, Par6 and aPKC) localize apically in radial precursors, along with cadherin complexes. Cdc42 is required for maintenance of radial precursor fate, by directing the apical localization of these and other proteins, including F-actin. In vertebrates, centrosomal proteins including LIS1, Nde1, and DCLK play a role in regulating mitotic spindle orientation and cell fate during neurogenesis, along with G-proteins, probably by regulating astral microtubule and F-actin cytoskeletal interactions and rearrangement.
Figure 1.4 *Overview of receptor tyrosine kinases induced signaling pathways.* Illustrating the multiple signaling pathways that can be activated downstream of RTKs such as FGFR, EGFR, PDGFR, Trk and ErbBR, in a cell-specific context, with some receptor-specificity. Kinases, phosphatases and other enzymes bind phosphotyrosine residues on activated receptors, either directly or via adaptor proteins such as Grb2, Gab1, FRS-2 and Crk. ERK-MAPK and PI-3K signaling is activated by Ras, downstream of activator complex including adaptor proteins, SOS, Gab1, Src and SHP-2. MAPKs (ERK, p38 and Jnk) regulate nuclear targets and gene transcription, including transcription factors that regulate survival, proliferation and differentiation, as well as proteins that potentiate or antagonize Ras-Mek-ERK signaling. The PKC-calcium signaling pathway is also activated downstream of PLC-γ signaling, and Ras signaling can also lead to activation of RhoGTPases in some cellular contexts.
Figure 1.5: Neurocardio-facial-cutaneous (NCFC) syndromes are caused by mutations in the Ras-MEK-ERK signaling pathway. A family of related disorders sharing overlapping phenotypes such as cardiac defects, cranio-facial abnormalities and neurological deficits has recently been classified based on causal mutations in genes encoding components of the Ras-MEK-ERK signaling pathway. Approximately, 50% of Noonan Syndrome (NS) cases are due to a spectrum of activating mutations in the gene encoding SHP-2, 30% of cases are caused by SOS mutations, and a few cases have been linked to K-Ras and Raf-1 mutations. Over 90% of Costello mutations are caused by G12H-Ras V/D mutations. The majority of CFC syndromes are caused by activating Braf mutations, as well as K-Ras and MEK1/2 mutations. LEOPARD syndrome is caused by gain-of-function but not activating SHP-2 mutations. Neurofibromatosis is caused by NF-1 loss-of-function mutations.
Figure 1.6: *Lfc* protein structure and regulatory domains. The Dbl-family Rho-guanine exchange factor Lfc is characterized by a classic dbl-homology (DH) domain which mediates its primary catalytic functions, in pair with the plecstrin homology (PH) domain which facilitates substrate targeting and binding. Important negative regulatory interactions are mediated at the n-terminus: the C1 domain, which is required for Lfc binding to microtubules and nearby residues which are required for binding by tctex-1, a negative regulator of Lfc. The activity of Lfc is also regulated at the post-translational level by PKA and ERK.
Chapter 2: Experimental Procedures

a) Cell culture
Primary cortical precursor cultures: As described previously (Ménard et al., 2002; Barnabé-Heider & Miller, 2003), cortices from E11-12.5 CD-1 embryos were dissected in ice-cold HBSS, taking care to remove the meninges. The dorsal part of the telencephalon was transferred into ice cold neurobasal media containing 40ng/mL FGF2 (BD Sciences or Promega), 2% B27 supplement (Gibco), 1% Pen/Strep (Gibco) and 500µM L-glutamine (Gibco). The cortices were mechanically dissociated to single cells and plated on 4 well chamber slides (Lab-Tek) coated with 1% poly-D lysine (BD Sciences or Sigma) and 2% laminin (BD Sciences) for immunocytochemistry. 1.25 x 10^5 cells were plated per well in a final volume of 500 µL of media. For Western blot analyses, 1 to 2 x 10^6 cells were plated per well (6 well dish) in 1.5 mL media. For long term cultures, half of the media was removed every other day and replaced with fresh media.

NIH3T3 and HEK293 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 120 µg/mL penicillin, 200 µg/mL streptomycin sulfate, and 600 µg/mL glutamine.

b) Growth factor and Inhibitor treatments
CNTF (Cedarlane Laboratories) was used at 50ng/ml. AG490 (Calbiochem) and PD98059 (BIOMOL Research Laboratories) were used at concentrations of 5µM and 50µM in 0.75% DMSO respectively. ZVAD-FMK (Calbiochem) was used at a concentration of 100 µM in 0.5% DMSO.

c) Expression vectors
Expression plasmids used were pEF-EGFP (the kind gift of R. Kageyama, Kyoto; Paquin et al., 2005), pEF-GM (empty vector; from R. Kageyama), and pSUPER-scrambled shRNA or pSHN-scrambled as controls. For the SHP-2 constructs, C-terminal HA-tagged forms of human wildtype (WT)-SHP-2, D61G-SHP-2 or dominant-negative (ΔP-SHP-2) (Tang et al., 1995) were subcloned from pBluescript into the pEF-GM vector using SalI/NotI restriction
sites. A C-terminal HA-tagged form of wildtype (WT), dominant-negative (ΔP – lacking 30 amino acids in the phosphatase domain) human SHP-2 were subcloned into the pEF-GM vector using PCR. A forward primer containing a SalI restriction site, and a Kozac sequence (5’gtgctgaccttggccacct catgcctgcgaatg), and a reverse primer containing a HA-tag sequence and NotI restriction site (5’gtgccgctcagggcagctgtaatc) were used, and the resulting 2.1kb product subcloned in the pEF-GM vector at SalI/NotI sites. An activated form of SHP-2 (D61G SHP-2) was generated by PCR of an existing GST-SHP-2 cDNA (B. Neel) using the following primers (1, 2), and a 1.2kb BglII fragment was swapped with that of the WT SHP-2 construct to generate the c-terminal HA-tagged D61G-SHP-2 in pEFGM. cDNAs were sequenced after every plasmid preparation.

The shRNAs cloned in the pSuperiorRetroPuro vector (OligoEngine) were provided by B.G. Neel’s lab, and the sequences were:

SHP-2shRNA-1
5’ATCCCCGATTCAGAACACTGGGGACTTCAAGAGAGTCCCCAGTGTTCTGAACTTTTTTGGAAA3’

SHP-2shRNA-2
5’GATCCCCGAGTAACCCTGGAGACTTCTTCAAGAGAGAAGTCTCCAGGGTTACTCTTTTTGGAAA3’.

Lfc shRNA constructs provided by the Rottapel lab were generated by cloning the following hairpin sequences into the Bgl II and Hind III sites of pG-SHIN2 and the sequences are

LfcshRNA 1:
5’GATCCCCAACCCTTCAATGGGCTCCCATTTGAAGAGAGATTTCAATGGGACCTTGAAGGTTTTTTT-3’ and
5’AGCTAAAAAAAAACCTTCAATGGGCTCCCATTTGAAGCTCTTTGAAGGTTCTT-3’,

LfcshRNA 2:
5’GATCCCCCTGAGAACCTTGAAGATTATTTCAAGAGAATAATCTTCAAGGTGTTCTCA TTTTT-3’ and
shRNA constructs targeting Tctex-1 were made based on sequences published previously (Chuang et al., 2005) and provided by the Rottapel lab. The following hairpins were cloned into pG-SHIN2 as described above; 

Tctex-1shRNA-1:

5’-GATCCCCGTCAACCAGTGGACCACCTATTCAAGAGATAGTGGTCCACTGAGTTGACTTTTT-3’

and

5’-AGCTAAAAAGTCAACCAGTGGACCACCTATTCTCTTGAAATAGTGGTCCACTGAGCGGG-3’,

Tctex-1 2: 5’-GATCCCCGTGAAGTACCGCTAAGGATTCAAGAGATCCTTAGCGGTACTTCACCTTTTT-3’

and

5’-AGCCTAAAAAGGTACCGCTAAGGATTCTCTTGAAATAGTGGTCCACTGAGCGGG-3’.

A scrambled hairpin-expressing pG-SHIN2 construct was generated using the following sequences: 5’-GATCCCCGTTACACACCGCAAGTTCTTCAAGAGAAGTTGCGGTGTAACCTTTTT-3’

and

5’-AGCTAAAAAGGTACCGCTAAGGATCTCTTGAAATAGTGGTCCACTGAGCGGG-3’.

pSHIRAZ constructs expressing shRNA sequences identical to those described above were created by the Rottapel lab by excising eGFP and cloning DsRed2 into the AgeI and NotI sites of pG-SHIN2.

d) Cell culture transfections

For transfections of precursors, within two hours of plating, cells were treated with Fugene 6 (Roche) mixing a total of 1µg DNA plasmid , 1.5 µl Fugene6 and 100µl OptiMEM (Invitrogen) and incubating for 35-45 minutes at ambient temperature before adding to the cells, as outlined by the manufacturer. For the SHP-2 experiments, plasmids that did not contain a bi-cistronic eGFP expression cassette or HA-tag were co-transfected with pEF-
eGFP using the following ratios: 0.33 µg pEGFP and 0.66 µg of pEF-GM, pSUPER-scrambled shRNA, pSUPER-SHP-2 shRNA1 or shRNA 2.

Transient transfections of NIH3T3 or HEK293 cells were performed at 80% confluency using Lipofectamine 2000 and Opti-MEM medium as outlined by the manufacturer (Invitrogen).

e) In utero electroporation

In utero electroporation was performed as described (Barnabé-Heider et al., 2005; Paquin et al., 2005) with E13/E14 CD1 or Tα1:nlacZ (Gloster et al., 1994; 1999) mice, injecting a nuclear EGFP expression plasmid driven from the EF1α promoter (pEF-EGFP) (as above) at a 1:3 ratio with pSUPER-shRNA1, pEF-D61GSHP-2, pEF-ΔPSHP-2, pEF-GM (empty vector), pSUPER-scrambled as a control (5 µg/µl) or pSHIN-scr, -LfcshRNA1/2 or -TctxshRNA1/2 (3.7 ug/ul) and 0.05% trypan blue as a tracer. When pSHIN- and pSHIRAZ-shRNAs were co-injected, the final DNA concentration was 6.4ug/ul. The square electroporator CUY21 EDIT (TR Tech, Japan) was used to deliver five 50ms pulses of 40-50V with 950ms intervals per embryo. Brains were dissected in ice-cold HBSS, fixed in 4% PFA at 4ºC overnight, cryoprotected and cryosectioned coronally at 16µm.

f) BrdU injections and Immunostaining

Pregnant CD1 mice were injected with Bromodeoxyuridine (BrdU) (Sigma) dissolved in PBS at a dose of 50mg/kg one day post-electroporation.

g) Immunocytochemistry and analysis

In general, for immunostaining of cultured cells, cells were gently washed with HBS or PBS and fixed with 4% PFA for 15 minutes, permeabilized with 0.2% NP-40 for 5 minutes, and blocked in 0.5% BSA and 6% normal goat serum (or other serum appropriate for secondary antibody) in HBS or PBS for 1-2 hours at room temperature. Slide covers were removed at this point, and slides were incubated with primary antibody at 4ºC overnight, with secondary antibodies at room temperature for 1 hour, counterstained with Hoechst 3325 (1:2000), Sigma-Aldrich), and mounted with GelTol (Fisher) or Permafluor (Labvision)
*For detection of proteins at the mitotic spindle, a fixation/extraction procedure was employed prior to the blocking step. Briefly, cultured cortical precursors were treated for 1 minute at room temperature in pre-extraction buffer (0.1% Triton-X-100, 80 mM PIPES, 5 mM EGTA, 1 mM MgCl₂, 25 mM Hepes pH 6.94), washed once in PBS and then placed in fixation/extraction buffer (2.7% formaldehyde, 0.5% Triton-X-100, 0.25% glutaraldehyde, 80 mM PIPES, 5 mM EGTA, 1 mM MgCl₂, 25 mM Hepes pH 6.94) for 15 minutes at room temperature.

For immunostaining of tissue sections, sections were post-fixed with 4% PFA, blocked and permeabilized with 10% BSA and 0.3% Triton-X, and then with the M.O.M. blocking kit (Vector Laboratories). Sections were incubated with primary antibodies at 4ºC overnight, with secondary antibodies at room temperature for 45 minutes, counterstained with Hoechst 33258 (1:2000, Sigma-Aldrich) and mounted with GelTol (Fisher).

*For Tbr2 immunostaining, an antigen presentation step was included where sections were boiled for 1 minute in 0.01M citrate buffer prior to blocking and permeabilization.

**For BrdU staining, sections were dried for 15min, incubated in 2N HCL for 30 minutes at 37º C and washed in 0.1M borate buffer for 5 minutes prior to blocking as described above. A ki67 immunostaining was performed first as described above, before incubating sections with the Alexa-Fluor conjugated anti-BrdU antibody (1:50) at room temperature for 1-2 hours.

Primary antibodies used for immunolabeling were mouse anti-GFP (1:1000, Molecular Probes), rabbit anti-GFP (1:400, Chemicon), chicken anti-GFP (1:1000, Molecular Probes), mouse and rabbit anti-HA (1:400, Covance), rabbit anti-SHP-2 (1:500, Santa Cruz Biotechnology), mouse anti-nestin (1:400, Chemicon), mouse and rabbit anti-βIII tubulin/Tuj1 (1:1000, Covance), rabbit anti-GFAP (1:800, Chemicon or 1:1000, Axell), mouse anti-HuD (1:200, Molecular Probes), rat anti-CD44 (1:200, Chemicon for tissue sections or 1:400, BD-Pharmingen for cultured cells), rabbit anti-phosphoSTAT3-Tyr705 (1:200 Cell Signaling Technology), rabbit anti-phosphoERK-Thr185/Tyr187 (1:500, Cell Signaling Technology; 1:500, Promega), mouse anti-Ki67 (1:200, BD Bioscience). sheep
anti-Lfc (1:200, Bakal et al., 2005), goat anti-doublecortin (1:100, Santa Cruz), mouse anti-Ki67 (1:200, BD Bioscience), rabbit anti-BrdU Alexa-546 conjugate (1:100, Molecular Probes), mouse anti-Pax6 (1:400, DSHB), rabbit anti-Tbr2 (1:300, Chemicon), rabbit anti-phospho-Histone H3 (1:1000, Upstate), anti-alpha tubulin (1:200, Abcam), mouse anti-gamma tubulin (1:200, Santa Cruz), anti BrdU-conjugated with Alexa Fluor 546 (1:50, Molecular Probes). The Pax6 antibody developed by Dr. Kawakami was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA, 52242.

Secondary antibodies used for immunocytochemistry were indocarbocyanine (CY3)-conjugated goat anti-mouse or anti-rabbit IgG (1:400), FITC-conjugated anti-mouse or anti-rabbit IgG (1:100, Jackson Immuno Research Laboratories), Alexa Fluor 555 goat anti-rat (1:1000, Molecular Probes), Alex Fluor 350 goat anti-mouse (1:500, Molecular Probes), DTAF-conjugated streptavidin (1:2000), and CY3-conjugated streptavidin (1:1000, Jackson ImmunoResearch Laboratories). Alexa Fluor 555-, Alexa Fluor 488-, or Alexa Fluor 565-conjugated goat anti-mouse or anti-rabbit IgG (1:1000, Molecular Probes), Alexa Fluor 488-, or Alexa Fluor 555-conjugated donkey anti-sheep, anti-mouse or anti-rabbit (1:1000, Molecular Probes), and Cy3-conjugated donkey anti-goat(1:1000, Jackson ImmunoResearch Laboratories).

**h) Microscopy and quantification**

Microscopy of cultured cortical precursors was carried out using a Zeiss Axioplan2 upright microscope equipped with fluorescence optics. Analysis of cultured cortical precursors was performed by scoring over 200 transfected cells per condition per experiment in at least eight randomly selected fields spanning the culture well. In clonal studies, 70 to 120 clones were analyzed per condition per experiment. Clones were defined as groups of GFP-positive cells in close proximity to each other that were well-separated from any other GFP-positive clusters, but that were not part of distinct unlabeled clusters. Digital image acquisition and analysis was performed using Northern Eclipse software (Empix Inc.) with a Sony XC-75CE CCD video camera. For electroporated brains, coronal sections were analyzed using a Zeiss
Pascal confocal microscope and the manufacturer’s software. A total of three to four sections were analyzed per animal by taking up to three 8-10 μm z-stack pictures to cover the electroporated ventricular zone, subventricular zone and cortical mantle of each coronal section with a 40X objective and compared to equivalent sections in littermate counterparts. For mitotic spindle measurements, images of immunostained sections were acquired with a Zeiss Axiovert 200 inverted microscope equipped with a Hamamatsu Orca AG CCD camera and spinning disk confocal scan head. 12 μm Z-stacks were taken at 0.3 μm thickness for each optical section and images were analyzed with Volocity (Improvision) and ImageJ (NIH) software programs. Transfected, mitotic cells in metaphase, anaphase and telophase were identified by phospho-histone H3, gamma-tubulin and Hoechst nuclear staining. The angle between the ventricular surface and the line between the centrosomes was measured. An angle between 0-15 degrees was scored as a vertical division. A total of 95 cells from eight Lfc shRNA-transfected brains and 61 cells from eight scrambled shRNA-transfected brains were scored in three separate experiments. In all graphs, error bars indicate Standard Error of the Mean (SEM), and statistics were performed using Students t-test or ANOVA, as appropriate.

For quantitation of total cells, neurons and astrocytes in SHP-2D61G/+ mice (Araki et al., 2004), mice were bred on a 129J/B6 background, and brains harvested and processed as described above at postnatal day 0 and 2. In blind analyses, 3-4 coronal tissue sections were selected from the same rostral-caudal extent (based upon hippocampal and cortical morphology) in brains of D61G/+ and +/+ littermates and age-matched non-littermates, and cell counts were made from defined regions in the dorsal cortex and dentate gyrus from sections processed by immuhistochemistry for HuD, NeuN, and GFAP, and counterstained for Hoechst. Digital images were acquired and analyzed using Northern Eclipse software and a Sony XC-75CE CCD camera as described above. In all graphs, error bars indicate Standard Error of the Mean (SEM), and statistics were performed using Students t-test or ANOVA, as appropriate.
i) Clonal analysis

For clonal analysis in the SHP-2 project, cortical precursors were transfected as above, and cultures were immunostained at 4 or 6 DIV for EGFP, βIII-tubulin and GFAP. In some experiments, CNTF was added at 2 DIV, and some experiments were carried out in the presence of the pan-caspase inhibitor ZVAD. In the Lfc and Tctex-1 project, similar cultures were analyzed at 2 or 3 DIV, in the absence of any exogenous growth factors or pharmacological inhibitors. In all of these experiments, clusters of isolated, EGFP-positive cells were then analyzed for their composition, size, and cell death. Clones containing at least one βIII-tubulin positive neuron but no astrocytes were classified as neuronal clones, those containing at least one GFAP-positive astrocyte, but no neurons, as astrocyte clones, and those containing both neurons and astrocytes as bipotent clones. Undifferentiated clones were also counted.

j) Western blot analysis and biochemistry

To prepare cortical tissue lysates, cortices were dissected from CD1 embryonic mice at specific gestational time-points. For both cortical tissues, HEK 293 and NIH 3T3 cells, material was rinsed twice in ice-cold phosphate-buffered saline (PBS) and lysed in NP-40 lysis buffer supplemented with 1 mM sodium vanadate and inhibitor cocktail. 30-50ug total protein lysate was separated by SDS-PAGE, transferred to nitrocellulose membrane, and immuno-blotted with Blots were developed by Enhanced Chemiluminescence (GE Healthcare). Antibodies used were rabbit anti-SHP-2 (1:500, Santa Cruz Biotechnology), and rabbit anti-ERK (1:5000, Santa Cruz Biotechnology), sheep anti-Lfc (1:200, Bakal et al., 2005), rabbit anti-Tctex-1 (1:500, Proteintech). The secondary antibody used for biochemistry was HRP-conjugated goat anti-mouse, anti-rabbit or anti-sheep (1:5000, Biorad). Blots were developed by Enhanced Chemiluminescence (GE Healthcare).
Chapter 3: SHP-2 Regulates Cell Fate Decisions in the Normal and Noonan Syndrome CNS

The data presented in this chapter was published in April 2007 in a research article entitled:


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Author contributions:
A.S.G. performed all aspects of the majority of the experiments presented in this chapter, with the exception of the following: O. F. performed the experiments related to testing the efficiency of SHP-2 shRNA-mediated knockdown in 3T3 cells (Fig 1d) and helped with the phosphatase assays (unpublished, mentioned in discussion). T. A. from B.G.N’s lab organized breeding and shipment of D61G/+ mice and brains, and provided the D61GSHP-2 cDNA. R.C. also from B.G.N.’s lab designed the unpublished and untested SHP-2 shRNAs. B.G.N, D.R.K and F.D.M contributed intellectually and to the writing of the manuscript.

3.1 SUMMARY

Within the developing mammalian CNS, growth factors direct multipotent precursors to generate neurons versus glial cells, a process that, if perturbed, might lead to neural dysfunction. Here, we demonstrate that the growth factor-regulated SHP-2 protein tyrosine phosphatase is essential for normal cortical cell fate determination, and that a mutation resulting in constitutive activation of SHP-2 in Noonan Syndrome, a human syndrome
associated with mental retardation and learning disabilities, causes perturbations in this developmental process. Specifically, genetic knockdown of SHP-2 in cultured cortical precursors or in the embryonic telencephalon inhibited basal neurogenesis and caused enhanced and precocious astrocyte formation. Conversely, expression of a Noonan Syndrome SHP-2 isoform enhanced MEK-ERK signaling to promote neurogenesis and negatively-regulated the gp130-JAK-STAT pathway to inhibit gliogenesis. Neural cell fate decisions were similarly perturbed in a mouse knock-in model that phenocopies human Noonan Syndrome. Thus, SHP-2 instructs precursors to make neurons and not astrocytes during the neurogenic period, and perturbations in the relative ratios of these two cell types following constitutive SHP-2 activation may contribute to the cognitive impairments in Noonan Syndrome patients.

3.2 BRIEF INTRODUCTION AND RATIONALE

Development of the cerebral cortex is achieved through a heterogeneous pool of precursor cells that sequentially generate neurons and glial cells. Emerging evidence indicates that while intrinsic cues are important in cortical cell fate decisions, the developmental availability of growth factors determines the appropriate timed genesis of neurons versus astrocytes (Miller and Gauthier, 2007). In particular, appropriate early neurogenesis requires receptor tyrosine kinase-mediated activation of a MEK-ERK-C/EBP pathway (Ménard et al., 2002; Paquin et al., 2005), while the later onset of astrocyte formation requires activation of the gp130-JAK-STAT pathway (Bonni et al., 1997; Johe et al., 1996) by neuron-derived cardiotrophin-1 (Barnabé-Heider et al., 2005), a cytokine that is not expressed in the cortex until late embryonic timepoints (Oppenheim et al., 2001; Barnabé-Heider et al., 2005).

Implicit to any model where growth factors define neural cell fate is the assumption that multipotent precursors are able to respond to both neurogenic and gliogenic factors even at timepoints when those cell types are not normally generated. While cortical precursors become increasingly responsive to gliogenic cytokines as development proceeds (Miller and Gauthier, 2007), it is nonetheless clear that many cortical precursors are capable of making astrocytes even during the neurogenic period if exposed to gliogenic cytokines (Morrow et al., 2001; Barnabé-Heider et al., 2005). Mechanisms must therefore exist to ensure that
precursors enact only one cell fate decision even when exposed to multiple, contradictory growth factor cues. Previous work indicates that such biasing mechanisms do exist in neural precursors since growth factors that promote gliogenesis decrease neurogenesis (Shah et al., 1996; Bonni et al., 1997; Yanagisawa et al., 2001; Barnabé-Heider et al., 2005), and the neurogenic growth factor PDGF can, in some conditions, “override” the gliogenic effect of CNTF in cultures of cortical precursors (Park et al., 1999). Here, we have asked about potential growth factor-regulated signaling proteins that might mediate these effects by promoting one neural cell fate while at the same time inhibiting another, and have identified SHP-2.

SHP-2 is a growth factor-regulated protein tyrosine phosphatase that is expressed in the central nervous system (Reeves et al., 1996; Servidel et al., 1998), and that has been shown in many cell types to cells to regulate both the MEK-ERK and the gp130-JAK-STAT pathways (Neel et al., 2003). With regard to the MEK-ERK pathway, SHP-2 is recruited to many receptor tyrosine kinases upon activation, and is then essential for sustained ERK activation (Neel et al., 2003). For example, in cortical neurons, SHP-2 is a positive regulator of TrkB-mediated ERK activation (Easton et al., 2006). SHP-2 mediates this positive regulation primarily by dephosphorylating and inactivating proteins that negatively regulate Ras signaling (Neel et al., 2003). SHP-2 is also recruited to the activated gp130 receptor, which in turn activates SHP-2 to negatively regulate the gp130-JAK-STAT pathway (Ohtani et al., 2000; Lehmann et al., 2003; Ernst and Jenkins, 2004; Clahsen et al., 2005). Thus, SHP-2 functions as a positive regulator of growth factor-mediated MEK-ERK activation and as an inhibitor of JAK-STAT signaling, making it an ideal candidate for promoting neurogenesis and at the same time inhibiting gliogenesis. Further support for the idea that SHP-2 may regulate neural development comes from the genetically-defined human Noonan Syndrome. In Noonan Syndrome, which occurs in 1 in 1000 live births, children present with cardiac defects, craniofacial abnormalities, and short stature (Noonan, 1994), and a large percentage (1/3 to 1/2) exhibit learning disabilities and mental retardation (Noonan, 1994; Yoshida et al., 2004; Lee et al., 2005). In about fifty percent of cases, Noonan Syndrome is caused by missense mutations in the human PTPN11 (SHP-2) gene, often resulting in expression of a SHP-2 protein with increased basal or stimulated phosphatase activity (Tartaglia et al., 2001; Keilhack et al., 2005). While this genetic syndrome can be modelled
in the mouse by knocking-in a Noonan Syndrome SHP-2 allele (Araki et al., 2004), it is not yet known whether this mouse model displays neural perturbations that might explain the human cognitive deficits.

Here, we have asked whether SHP-2 acts as a growth factor-regulated switch to instruct cortical precursors to adopt one fate versus another, and whether this function might be perturbed in Noonan Syndrome.

3.3 RESULTS

3.3.1 SHP-2 is necessary for cultured cortical precursor cells to generate neurons

To elucidate the mechanisms responsible for regulating the generation of neurons versus astrocytes from multipotent precursors, we examined primary murine E12 cortical precursor cells, a system we have previously characterized in detail (Toma et al., 2000; Ménard et al., 2002; Barnabé-Heider and Miller, 2003; Barnabé-Heider et al., 2005). Upon plating in FGF2, these cortical precursors are virtually all dividing, nestin-positive cells that sequentially generate neurons and then glia; neurons are first observed after 1 day in vitro (DIV), while astrocytes and oligodendrocytes are first seen at 5-6 DIV. This increase in differentiated cells is accompanied by a depletion of proliferating precursors.

We have previously shown that neurogenesis in cortical precursors requires activation of the MEK-C/EBP signalling pathway (Ménard et al., 2002; Barnabé-Heider and Miller, 2003; Paquin et al., 2005), while astrocyte formation requires cardiotrophin-1-mediated gp130-JAK-STAT activation (Barnabé-Heider et al., 2005). Since the SHP-2 tyrosine phosphatase regulates both of these signalling cascades in many cell types, we asked whether it might regulate cell fate decisions in cortical precursors. Initially, we demonstrated that SHP-2 is expressed in differentiating cortical precursor cultures by Western blot analysis (Fig. 1a). Immunocytochemical analysis for SHP-2 and cell type-specific markers at 2 DIV (Fig. 1b) revealed that SHP-2 was expressed in both precursors and newly-born neurons in these cultures. We then characterized expression of SHP-2 in the embryonic cortex in vivo by immunocytochemistry. This analysis revealed that during the neurogenic period, at E12 and E15, SHP-2 was highly enriched in nestin-positive precursor cells of the ventricular/subventricular zones, and was expressed at lower relative levels in neurons in the cortical plate region (Fig. 1c). In contrast, by E17, which coincides with commencement of
the gliogenic period, the highest relative levels of SHP-2 were in the cortical mantle region in neurons, with relatively low expression in nestin-positive precursors of the VZ/SVZ. A similar expression pattern was observed at postnatal day 5 (Fig. 1c). Thus, SHP-2 is high in cortical precursors during the neurogenic period, with lower levels in cortical precursors during the gliogenic period.

Given this pattern of expression, we asked whether SHP-2 was necessary for the genesis of neurons from cultured cortical precursors. To ask this question, we utilized two different shRNA sequences to knock-down SHP-2 levels. Initially, we determined the efficacy of these two SHP-2 shRNAs by cotransfecting them along with an EGFP-expression plasmid into NIH 3T3 cells. Western blot analysis revealed that total SHP-2 levels were reduced at 48 to 72 hours posttransfection even though only approximately 30% of the 3T3 cells were transfected (Fig. 1d). Both shRNA vectors were effective when compared to a scrambled shRNA control, although shRNA1 was more effective than shRNA2 (Fig. 1d; data not shown).

Having demonstrated the efficacy of these shRNA vectors, we cotransfected them along with an EGFP-expression plasmid into freshly-plated cortical precursors to ask whether SHP-2 was necessary for appropriate neurogenesis. At the time of plating and transfection, the cultures are comprised only of proliferating cortical precursor cells. Immunocytochemical analysis of these transfected precursors at 2DIV for EGFP and SHP-2 (Fig. 1e) revealed that shRNA1 and shRNA2 both reduced SHP-2 levels in transfected precursors, although as seen in NIH 3T3 cells, shRNA1 was the more effective of the two (Fig. 1f; data not shown). In contrast, SHP-2 levels were significantly higher in 90% of EGFP-positive cells that were cotransfected with a point mutant of SHP-2 (D61G SHP-2, discussed below) (Fig. 1f). We then performed similar experiments and asked whether this shRNA-mediated knockdown of SHP-2 had any effect on neurogenesis by immunostaining the cells for the neuron-specific protein βIII-tubulin at 3DIV (Fig. 2a). Quantitative analysis revealed that both SHP-2 shRNA1 and shRNA2 significantly decreased the number of EGFP-positive transfected neurons (Fig. 2b). To confirm that this effect was due to a specific decrease in SHP-2 levels, we performed a rescue experiment using human SHP-2, which would not be targeted by the murine shRNAs. Precursors were cotransfected with plasmids encoding HA-tagged human SHP-2, and SHP-2 shRNA1, and then analyzed 3 days
later for βIII-tubulin. This analysis revealed that the human SHP-2 completely rescued the decrease in neurogenesis caused by murine SHP-2 shRNA (Fig. 2b).

While these data support the idea that SHP-2 is required for neurogenesis, alternative explanations are that SHP-2 is necessary for proliferation of cortical precursors, or survival of cortical precursors and/or newly-born neurons. To address these possibilities, precursors were cotransfected with plasmids encoding EGFP and SHP-2 shRNA1, shRNA2, or scrambled shRNA, and then were analyzed immunocytochemically at 2DIV for Ki67, to monitor proliferation, or cleaved caspase-3, to monitor apoptosis. Quantitative analysis revealed that the SHP-2 shRNAs had no significant effect on the proliferation (Fig. 2c) or apoptosis (Fig. 2d) of transfected cortical precursors. Counts of the number of uncondensed Hoechst-positive nuclei in these cultures confirmed that SHP-2 shRNA knock-down had no effect on cell survival (Fig. 2e). As a final control, we performed similar experiments in the presence of the pan-caspase inhibitor ZVAD; SHP-2 shRNA reduced the percentage of neurons in these cultures when apoptosis was inhibited (Fig. 2f). Thus, SHP-2 is not necessary for cortical precursor cell survival or proliferation, but is necessary for the genesis of cortical neurons.

### 3.3.2 SHP-2 inhibits cytokine-mediated genesis of astrocytes in cortical precursor cultures

Since SHP-2 has been reported to negatively regulate the JAK-STAT pathway, we asked whether it also regulates astrocyte formation. To do this, we took advantage of the finding that astrogenesis can be prematurely induced in cultured precursors by adding exogenous cytokines such as CNTF or CT-1 (Bonni et al., 1997; Johe et al., 1996; Ochiai et al., 2001). Specifically, precursors were transfected with SHP-2 shRNA1, shRNA2, or the scrambled shRNA, induced with CNTF at 2 DIV, and then analyzed immunocytochemically at 6 DIV for the astrocyte marker GFAP (Fig. 3a). Quantitative analysis of these experiments revealed that a knockdown of SHP-2 with either shRNA1 or shRNA2 robustly increased the number of GFAP-positive astrocytes generated in response to CNTF relative to the scrambled shRNA controls (Fig. 3b). Similar results were obtained when cultures were immunostained for the early astrocyte/astrocyte precursor marker CD44 (Fig. 3c) (Liu et al., 2004; Barnabé-Heider et al., 2005), indicating that SHP-2 normally functions in cultured cortical precursors to inhibit cytokine-mediated astrogenesis.
To ask whether this inhibition of astrogenesis required the phosphatase activity of SHP-2 (this protein can also function as an adaptor), we used a dominant-negative SHP-2 mutant, ΔP-SHP-2, that has impaired substrate binding and phosphatase activity due to a 30 nucleotide deletion in the phosphatase domain (Tang et al., 1995). Precursors were cotransfected with plasmids encoding EGFP and ΔP-SHP-2, exposed to CNTF at 2 DIV, and then immunostained for GFAP at 6 DIV. This analysis revealed that, as seen with the SHP-2 shRNAs, ΔP-SHP-2 significantly enhanced astrocyte formation in response to CNTF (Fig. 3d). We therefore asked whether the neurogenic effects of SHP-2 also required its phosphatase activity; cortical precursors were transfected with ΔP-SHP-2, and 3 days later were immunostained for βIII-tubulin. This analysis revealed that expression of ΔP-SHP-2 had no effect on the numbers of neurons that were generated (Fig. 3e), suggesting that astrogenesis, but not neurogenesis, required the phosphatase activity of SHP-2.

### 3.3.3 SHP-2 instructs cortical precursors to generate neurons versus astrocytes

While these data suggest that SHP-2 promotes neurogenesis and inhibits gliogenesis, it could be doing so either by instructing precursors to make neurons rather than glia, or by regulating the number of progeny generated by previously committed progenitors. To distinguish these possibilities, we performed clonal analysis under conditions where both neurons and astrocytes were generated. If SHP-2 acts instructively, then it would regulate the number of clones containing neurons and/or astrocytes. If, conversely, SHP-2 regulated the number of differentiated progeny from biased precursors, then the numbers of neuronal versus glial clones would stay the same, but the numbers of differentiated cells in those clones would change. To perform these experiments, cortical precursors were transfected at very low densities with EGFP and SHP-2 shRNA1 plasmids, CNTF was added at 2 DIV, and cultures were immunostained at 4 DIV for EGFP, βIII-tubulin and GFAP. Alternatively, cells were transfected and cultured in the absence of CNTF. In either case, isolated, EGFP-positive clones of cells were then analyzed for their composition (Fig. 3f); clones containing at least one βIII-tubulin positive neuron but no astrocytes were classified as neuronal clones, those containing at least one GFAP-positive astrocyte, but no neurons, as astrocyte clones, and those containing both neurons and astrocytes as bipotent clones. Clones containing no cells expressing either of these markers were also observed. This analysis revealed that, in
the presence of CNTF, SHP-2 knockdown decreased the number of neuronal clones while at the same time increasing the number of astrocyte clones (Fig. 3g). Bipotent clone numbers were unchanged (4-5% with and without SHP-2 shRNA; p>0.05), and clone size was unaffected (p>0.05). In the absence of CNTF, SHP-2 knockdown reduced the number of neuronal clones, and caused premature genesis of astrocyte clones at both 4 and 6 DIV (Fig. 3h). Thus, endogenous SHP-2 instructs precursors to generate neurons at the expense of glia.

This triple-label analysis also revealed one unexpected result; while control transfected cells never coexpressed βIII-tubulin and GFAP, SHP-2 knockdown caused the appearance of a small number of these apparently "confused" cells when CNTF was present in the cultures (Fig. 3i). Because of this, we ensured that the decrease in neuronal clones that we observed was not due to the death of neurons by performing similar experiments in the presence of ZVAD. Immunocytochemical analysis confirmed that SHP-2 knockdown caused a decrease in neuronal clones whether or not apoptosis was inhibited with ZVAD (Fig. 3j). Thus, endogenous SHP-2 acts to ensure that precursors become neurons rather than astrocytes, and that they do not attempt to adopt both fates in response to extrinsic cues.

### 3.3.4 A Noonan Syndrome constitutively-activated SHP-2 mutant promotes neurogenesis and inhibits gliogenesis from cultured cortical precursors

In fifty percent of Noonan Syndrome patients, the genetic defects, including learning disabilities and mental retardation, are the result of a variety of point mutations in the gene encoding SHP-2 that cause it to be either constitutively activated, or more easily activated as a protein tyrosine phosphatase (Hof et al., 1998; Tartaglia et al., 2001; Fragale et al., 2004; Yoshida et al., 2004). D61G SHP-2 is one such mutation that is seen in approximately 10% of Noonan Syndrome patients (Yoshida et al., 2004). Since our data indicate that SHP-2 functions to promote neurogenesis and inhibit gliogenesis, we asked whether this genetic mutation would alter cortical precursor cell fate decisions. Freshly-plated cortical precursors were cotransfected with plasmids encoding the HA-tagged D61G SHP-2 mutant, HA-tagged WT SHP-2, or EGFP as a control. Immunocytochemistry and quantitation three days later (Fig. 4a,b) revealed that expression of D61G SHP-2 caused an increase in the percentage of transfected βIII-tubulin-positive neurons in these cultures. A similar increase was seen when WT SHP-2 was overexpressed in cortical precursors (Fig. 4b). We then asked whether this
increase in neuron number was due to increased proliferation and/or survival of newly-born neurons as opposed to enhanced neurogenesis. Immunocytochemical analysis for Ki67 at 2DIV (Fig. 4c) revealed that D61G SHP-2 expression did not increase proliferation, but instead, modestly decreased it, consistent with the enhanced neurogenesis that we observed. Analysis of Hoechst-positive apoptotic nuclei (Fig. 4d,e) demonstrated that expression of either WT- or D61G SHP-2 had a very small but significant effect on cell survival, enhancing it from approximately 90% to 95% (Fig. 4d). Immunocytochemical analysis revealed that this was not due to an effect on neuronal survival, which was over 95% with or without D61G SHP-2, but was instead due to an increase of approximately 6% in the survival of βIII-tubulin-negative cortical precursors (Fig. 4e). This small increase in precursor cell survival did not influence the observed increase in neurogenesis, since when similar experiments were performed in the presence of ZVAD, D61G SHP-2 still increased the percentage of neurons to a similar extent as without ZVAD (Fig. 4f). Thus, increased levels or activity of SHP-2 directly promote the differentiation of neurons from cortical precursors.

We then asked whether the Noonan Syndrome SHP-2 D61G mutant also perturbed gliogenesis. Cortical precursors were transfected as in the neurogenesis experiments, and were exposed to CNTF at 2 DIV. Immunocytochemical analysis for GFAP at 6 DIV revealed that the D61G SHP-2 mutant caused a significant decrease in the percentage of transfected, GFAP-positive astrocytes that were generated (Fig. 4g,h). In contrast, overexpression of WT SHP-2 had no effect on the percentage of astrocytes (Fig. 4h), suggesting that the enhanced protein tyrosine phosphatase activity of D61G SHP-2 was responsible for this effect.

Finally, we asked whether these alterations in differentiated cell number reflected a perturbation in the cell fate decisions made by individual cortical precursors by performing clonal analysis. Precursors were cotransfected with D61G SHP-2 and EGFP-expressing plasmids, exposed to CNTF at 2 DIV, and clones were then analyzed immunocytochemically at 6 DIV (Fig. 3k). This analysis revealed that D61G SHP-2 increased the number of neuronal clones and concomitantly decreased the number of astrocyte clones. In contrast, D61G SHP-2 did not affect the numbers of either bipotent clones (approximately 3% in both cases, p=0.2), and clone size was unaffected (p>0.05). Similar results were obtained in the presence of ZVAD, indicating that differences in cell survival were not responsible for these
findings (Fig. 3l). Thus, D61G SHP-2 functions within individual cortical precursors to promote neurogenesis while at the same time inhibiting cytokine-induced astrocyte formation.

3.3.5 SHP-2 is necessary to promote neurogenesis during embryonic cortical development in vivo

These data indicated that SHP-2 was necessary to promote the generation of neurons and at the same time inhibit the generation of astrocytes from cultured cortical precursors. To ask whether this was also true in vivo, we performed in utero electroporation to knock-down SHP-2 expression in precursors of the ventricular/subventricular zones (VZ/SVZ) in the embryonic cortex (Barnabé-Heider et al., 2005; Paquin et al, 2005). We have previously demonstrated that when in utero electroporation is performed at E13/14, after 1 day, all of the transfected cells reside in the VZ/SVZ and approximately 85% of these cells are proliferating (Paquin et al., 2005). In control brains many of these transfected cells differentiate into neurons over the next few days, which then migrate out of the VZ/SVZ and ultimately reside within the cortical plate. Later in development, at early postnatal periods, some of the transfected cells which remain in the VZ/SVZ adopt an astrocytic fate.

Initially, we used this system to ask whether SHP-2 was required for neurogenesis. Plasmids encoding a nuclear EGFP and SHP-2 shRNA1 were transfected into the embryonic cortex at E13/14, embryos were allowed to develop three or four days in utero, and the cortex was analyzed immunocytochemically for EGFP and the neuron-specific protein HuD. A plasmid containing a scrambled shRNA sequence was used as a control. An initial examination of the pattern of EGFP-positive cells revealed a robust difference between control and SHP-2 shRNA1-transfected brains; whereas approximately 40-60% of the control cells had migrated into the cortical mantle (which includes both the intermediate zone of migrating neurons and the cortical plate), only 20-30% of SHP-2 shRNA1-transfected cells were present in these regions, with the majority instead localized to the VZ/SVZ (Fig. 5a,b). Since neurons are born in the VZ/SVZ, and then migrate out to the cortical mantle, we asked whether this difference in localization reflected a difference in the number of neurons that were generated. Quantitative confocal microscopic analysis revealed that many of the control, scrambled shRNA-transfected cells were HuD-positive neurons at this timepoint,
while many fewer HuD-positive neurons were observed in brains transfected with SHP-2 shRNA1 (Fig. 5c,d). In both cases, these HuD positive neurons were all localized to the cortical mantle, either within the cortical plate, or were migrating through the intermediate zone toward the cortical plate, with no HuD-positive cells located within the VZ/SVZ. Immunostaining for the very early neuronal protein, βIII-tubulin confirmed that newly-born neurons were not present within the VZ/SVZ in either control or experimental brains, indicating that neurons born in both conditions had successfully migrated out of the VZ/SVZ. Thus, SHP-2 knockdown led to a decreased percentage of newly-born cortical neurons.

To rule out the possibility that this decreased neuronal number was due to enhanced cell death and/or altered proliferation, we analyzed adjacent cortical sections immunocytochemically for cleaved caspase-3 and Ki67, respectively. In control and SHP-2 shRNA1-transfected brains, analysis for cleaved caspase-3 throughout the entirety of the transfected region revealed only rare double-labelled cells (Fig. 5e; less than 10 cells per brain in both cases), indicating that, as seen in culture (Fig. 2d,e), SHP-2 knockdown had no effect on apoptosis. Similarly, confocal analysis revealed that the percentage of transfected Ki67-positive cells, which were located only in the VZ/SVZ, were similar in control and SHP-2 shRNA1-transfected brains at both 3 and 4 days post-electroporation (Fig. 5f,g). Thus, SHP-2 knockdown inhibited neurogenesis in the embryonic cortex, leading to a decrease in the number of neurons migrating out of the VZ/SVZ into the cortical mantle.

3.3.6 SHP-2 knockdown leads to premature gliogenesis in the embryonic cortex

Our clonal data indicated that endogenous SHP-2 instructs precursors to make neurons rather than glia, and ensures they adopt only one fate even in the presence of conflicting cues. We therefore asked whether endogenous SHP-2 was responsible for inhibiting gliogenesis during the embryonic neurogenic period, a timeperiod when low levels of gliogenic cytokines are present in the cortex (Derouet et al., 2004; Uemura et al., 2002). To perform these experiments, embryos were electroporated with plasmids encoding GFP and SHP-2 shRNA1 at E13/E14, and analyzed 3 or 4 days later by double-labelling for GFP and GFAP. This analysis revealed that at E16/17 (3 days post-electroporation), no GFAP-positive cells were detected in cortices transfected with control, scrambled shRNA, but a small number of double-labelled astrocytes were seen in the cortices transfected with SHP-2
shRNA1 (Fig. 6a). By E17/18, some GFAP-positive astrocytes were detected in control cortices, but none of these derived from transfected precursors in the 3 brains that were analyzed (Fig. 6b). In contrast, SHP-2 shRNA-transfected, GFAP-positive astrocytes were observed in the two electroporated brains that were examined (Fig. 6b). Thus, genetic knockdown of SHP-2 leads to precocious astrocyte formation during the late neurogenic period.

To more quantitatively assess this enhanced gliogenesis, we performed similar electroporations, but analysed the brains at P3, early during the gliogenic period. We then quantitated transfected GFAP-positive cells within the SVZ, where all of the transfected, GFAP-positive cells were localized (Fig. 6c). This analysis (Fig. 6d) revealed that in control brains, less than 5% of the transfected cells within the SVZ were astrocytes. In contrast, approximately 15% of the SHP-2 shRNA1-transfected cells in this region were GFAP-positive, indicating that, as seen in culture, SHP-2 normally regulates the onset and extent of astrogensis. Since our culture data also indicated that the phosphatase activity of SHP-2 was essential for its gliogenic effects, we performed similar experiments with ΔP-SHP-2. Quantitative analysis revealed that, as seen with SHP-2 shRNA1, ΔP-SHP-2 increased the percentage of GFAP-positive astrocytes that were generated by 2-3 fold (Fig. 6e). Similar results were obtained for CD44 (Fig. 6f). We also analyzed neurogenesis in the ΔP-SHP-2 transfected brains 4 days post-electroporation, and observed, as we had seen in culture, that this had no effect on the number of HuD-positive neurons that were generated (Fig. 6g), indicating a differential requirement of the phosphatase activity for astrogensis versus neurogenesis. Thus, SHP-2 is required to suppress early and inappropriate gliogenesis, potentially acting via its phosphatase activity to suppress the gliogenic gp130-JAK-STAT pathway.

### 3.3.7 SHP-2 knockdown in the embryonic period leads to postnatal perturbations in cortical neurons

While the perturbations in gliogenesis that we observed in the neonatal cortex following SHP-2 knockdown were localized to the SVZ, analysis of the SHP-2 shRNA1-transfected cells within the remainder of the cortex revealed additional perturbations. In particular, while the percentage of transfected cells within the SVZ versus the cortical layers

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was similar in cortices transfected with SHP-2 shRNA1 versus scrambled shRNA (Fig. 6h,i), cell location within the cortical layers differed significantly. Specifically, the majority (95%) of control transfected cells were present in cortical layers II and III, but approximately 65% of the SHP-2 shRNA1-transfected cells were randomly scattered throughout layers IV-VI (Fig. 6i). Since we had already established that all of the transfected, GFAP-positive astrocytes were located in the SVZ, then to characterize the identity of these misplaced cells, we performed immunocytochemical analysis for HuD and O4, the latter a marker for oligodendrocytes. As seen in control brains, all of the SHP-2 shRNA1-transfected, O4-positive oligodendrocytes were located within the SVZ at this age, and not within the cortical layers (data not shown). Instead, the large majority of the SHP-2 shRNA1-transfected cells within the cortical layers were positive for HuD, regardless of their location (Fig. 6j,k). Thus, by P3, the percentage of transfected cortical neurons was similar in brains transfected with scrambled shRNA versus SHP-2 shRNA1, but these neurons were inappropriately located within the cortex. Since these experiments involved transient transfections at E14, then these findings may indicate that the SHP-2 knockdown was less efficient at later timepoints, and that neurons were ultimately generated from transfected precursors between E17/E18 and P3 but then migrated inappropriately due to their late birthdate. Alternatively, these data may indicate that SHP-2 plays a direct role in the migration of newly-born neurons.

3.3.8 A Noonan Syndrome SHP-2 activated mutant promotes neurogenesis and inhibits gliogenesis in the developing cortex

To ask whether the SHP-2 D61G mutation that occurs in Noonan Syndrome would lead to cell fate perturbation in the embryonic brain as we had observed in culture, we used in utero electroporation to introduce it into cortical precursors of the embryonic telencephalon. Initially, we examined potential effects upon neurogenesis by electroporating brains at E13/14 with plasmids encoding nuclear EGFP and the D61G SHP-2 mutant or WT SHP-2, and analyzing them three or four days later. Quantitative analysis using confocal microscopy for HuD revealed a significant increase in the number of D61G SHP-2 transfected cells that were neurons four days post-electroporation (Fig. 7a). In particular, while approximately 20-25% of control cells expressed HuD, approximately 30-35% of the cells expressing D61G
SHP-2 were positive for this neuronal marker. However, although there was an increase in their number, the location was similar for both control and D61G SHP-2-expressing, HuD-positive cells, with the vast majority (>99%) present outside of the VZ/SVZ in the cortical mantle. To ask whether WT SHP-2 had the same effect, we electroporated it into the lateral ventricles of E13/14 Tα1:nlacZ mice that we have previously characterized extensively (Gloster et al., 1994; 1999; Paquin et al., 2005); these transgenic mice express a nuclear β-galactosidase from the early neuronal promoter Tα1 α-tubulin. Analysis 3 days later revealed that WT SHP-2 robustly increased the number of newly-born, Tα1:nlacZ-positive neurons that were generated (Fig. 7b). Thus, as seen in culture, ectopic expression of either the D61G SHP-2 mutant or WT SHP-2 enhanced neurogenesis in vivo.

We also asked whether the D61G SHP-2 mutant inhibited astrocyte formation in vivo, as it did in culture. Cortices were electroporated at E14 with either D61G or WT SHP-2, and then were analyzed immunocytochemically at P3 for expression of GFAP or CD44 (Fig. 7c,d). Quantitative analysis of these brains (Fig. 7e-g) revealed that D61G SHP-2 significantly reduced astrocyte formation; in control brains approximately 5-10% of the transfected cells were GFAP-positive, while in D61G SHP-2 brains, only 1-2% were astrocytes (Fig. 7c,e). Similar results were obtained with CD44 (Fig. 7d,g). As seen for the neurons, D61G SHP-2 did not affect the localization of these cells, but only their numbers; virtually all of the EGFP-, GFAP-positive cells were located within the VZ/SVZ, as we have previously documented (Barnabé-Heider et al., 2005; Paquin et al., 2005). In contrast to D61G SHP-2, WT SHP-2 had no effect on the numbers of transfected cells that became astrocytes (Fig. 7f). Thus, the Noonan Syndrome D61G SHP-2 mutant perturbs cell genesis in the embryonic cortex, promoting the genesis of neurons and inhibiting the genesis of astrocytes.

3.3.9 SHP-2 promotes neurogenesis via activation of the MEK-ERK pathway, and inhibits gliogenesis by directly suppressing the gp130-JAK-STAT pathway

Our data indicate that endogenous SHP-2 promotes neurogenesis and inhibits gliogenesis, and that the gliogenic suppression likely occurs via a phosphatase-dependent mechanism. Since SHP-2 is known to promote receptor tyrosine-kinase-mediated activation of the Ras-MEK-ERK pathway, and since we have previously shown that MEK activity is
both necessary and sufficient for neurogenesis (Ménard et al., 2002; Barnabé-Heider et al., 2003; Paquin et al., 2005), then we asked whether SHP-2 promoted neurogenesis via MEK. Initially, we transfected cortical precursors with plasmids encoding EGFP and SHP-2 shRNA1 or the D61G SHP-2 mutant, and then asked whether this altered activation of the MEK-ERK pathway by immunostaining with an antibody specific to phosphorylated, activated ERK (Fig. 8a). Quantitation of the percentage of transfected precursors that expressed phosphoERK at levels lower (Fig. 8b, left panel) or higher (Fig. 8b, right panel) than basal levels revealed that SHP-2 shRNA1 reduced and D61G SHP-2 increased phosphoERK activation. Thus, SHP-2 is essential for normal levels of activation of the MEK-ERK pathway in cortical precursors. We then asked if the increased MEK-ERK activation observed with D61G SHP-2 was responsible for its ability to promote neurogenesis; transfected precursors were treated with a pharmacological MEK inhibitor, PD98059, and then immunostained for βIII-tubulin 3 days post-transfection (Fig. 8c). We have previously shown that this compound specifically inhibits MEK activity in cortical precursors, and that perturbing MEK activity has no effect on precursor cell survival or proliferation (Barnabé-Heider and Miller, 2003). This analysis confirmed that inhibition of MEK decreased the number of neurons that were generated under basal conditions, and that the D61G SHP-2 mutant enhanced neurogenesis (Fig. 8c). Moreover, inhibition of MEK with PD98059 was able to completely block the enhanced neurogenesis seen in cells transfected with D61G SHP-2. Thus, SHP-2 regulates neurogenesis via the MEK-ERK pathway.

In contrast to neurogenesis, gliogenesis in cortical precursor cultures depends upon cytokine-mediated activation of the gp130-JAK-STAT pathway (Bonni et al., 1997; Johe et al., 1996; Barnabé-Heider et al., 2005), and in many cell types, SHP-2 binds to activated gp130 and negatively regulates this signaling pathway (Ernst and Jenkins, 2004). We therefore asked whether SHP-2 regulated gliogenesis via this pathway. Initially, we asked whether SHP-2 negatively regulated the gp130-JAK-STAT pathway by cotransfecting precursors with plasmids encoding EGFP and SHP-2 shRNA1, ΔP-SHP-2, or D61G SHP-2. Cells were then exposed to CNTF at 2 DIV for one additional day, and then analyzed for the activation of STAT3 by performing immunocytochemistry with an antibody specific for phosphorylated STAT3 (Fig. 8d). This analysis revealed that knockdown of SHP-2 levels or
inhibition of SHP-2 phosphatase activity increased the number of precursor cells expressing detectable levels of phosphoSTAT3 by approximately 2-fold (Fig. 8e), consistent with the increased gliogenesis (Fig. 3). Conversely, the D61G SHP-2 mutant decreased the number of cells expressing detectable phosphoSTAT3 (Fig. 8e), consistent with the observed decrease in gliogenesis (Fig. 4).

These experiments indicated that SHP-2 was regulating the gp130-JAK-STAT pathway by dephosphorylation at or above the level of STAT3. To ask whether SHP-2 mediated its effect further upstream at the level of JAK or gp130, we determined whether the enhanced gliogenesis seen upon SHP-2 knockdown was dependent upon JAK activation. To perform these experiments, precursors were cotransfected with plasmids encoding EGFP and shRNA1, were induced with CNTF at 2 DIV for an additional four days with or without the pharmacological JAK inhibitor AG490. We have previously demonstrated that this inhibitor specifically blocks JAK activity in cortical precursors, and that it has no effect on survival or neurogenesis, but specifically inhibits astrocyte genesis (Barnabé-Heider et al., 2005). Immunocytochemistry for GFAP confirmed that inhibition of JAK activity blocked CNTF-mediated gliogenesis, and that SHP-2 shRNA1 enhanced the generation of astrocytes (Fig. 8f). Importantly, JAK inhibition also completely blocked the increased gliogenesis observed with SHP-2 shRNA1 (Fig. 8f). Thus, endogenous SHP-2 inhibits astrocyte formation by negatively regulating the gp130-JAK-STAT pathway.

3.3.10 A Noonan Syndrome mouse model displays perturbed cell genesis in the neonatal forebrain and hippocampus

While these findings suggest that the constitutive activation of SHP-2 seen in Noonan Syndrome could cause abnormal neural development, these were overexpression studies, whereas in Noonan Syndrome patients only one copy of the mutant allele is present. To overcome this limitation, we examined the brains of a Noonan Syndrome mouse model carrying one copy of the D61G SHP-2 that was knocked into the endogenous SHP-2 locus. This mouse phenocopies the human disorder (Araki et al., 2004), but the nervous system has not yet been examined. We therefore analyzed the cortex and hippocampus of newborn Noonan Syndrome mice, at a time when astrogenesis had just started (Fig. 9a). Initially, we examined coronal sections through the dentate gyrus of SHP-2 D61G/+ mice versus their
wildtype littermates and age-matched non-littermates (Fig. 9a, region 1). These sections were immunostained for the astrocyte marker GFAP (Fig. 9b) or the neuronal marker HuD, and counterstained with Hoechst 33258 (Fig. 9b) to allow quantitation of total neurons, astrocytes, cell numbers, and tissue area. These parameters were then quantitated in 3-4 coronal sections at the same rostral-caudal level of the dentate gyrus (Figs. 9a,b). This analysis revealed significant differences between the hippocampus of wildtype versus Noonan syndrome mice. Counts of total Hoechst-positive nuclei revealed that cell density was slightly, but significantly increased (~ 6%) in the Noonan syndrome mice (Fig. 9c). Quantitation of HuD-positive neurons also revealed a small, but significant increase in both the density of neurons, and in the percentage of neurons (Fig. 9e) in this same area. However, the most robust phenotype was revealed when GFAP-positive astrocytes were quantitated; both the density and the percentage of total cells that were astrocytes were decreased by 2.5-3 fold in the Noonan syndrome hippocampus (Fig. 9b,d).

Similar changes were seen in the dorsal cortex (Fig. 9a, region 2) of wildtype versus Noonan syndrome brains that were analyzed at postnatal day 2. This analysis revealed perturbations in the cortex similar to those seen in the hippocampus. The density of total cells displayed a small but significant increase (Fig. 9f,i), and both the density and the percentage of cells expressing the neuron-specific protein NeuN-positive also showed a small but significant increase (Fig. 9f,j). Moreover, quantitation of the number of GFAP-positive cells within the subventricular zone demonstrated an approximately 2-fold decrease in the density and percentage of astrocytes in the Noonan Syndrome versus wildtype cortex (Fig. 9g,k). In addition, the intensity of GFAP immunostaining in the glia limitans at this level was also reduced in the neonatal Noonan Syndrome brains (Fig. 9h). Thus, in both the dorsal cortex and hippocampus of neonatal Noonan Syndrome mice, there is a large decrease in astrocytes and a small but significant increase both in total cellular and neuronal density, alterations that may contribute to further perturbations during circuit formation, and that ultimately could lead to cognitive dysfunction.

3.4 CONCLUSIONS:

In summary, the data presented in this chapter support three major conclusions. First, the experiments using SHP-2 shRNA indicate that endogenous SHP-2 is essential for the normal
genesis of cortical neurons and astrocytes both in culture and within the environment of the embryonic cortex. Genetic knockdown of SHP-2 leads to a delay in neurogenesis and ultimately a perturbation in neuronal location. It also causes inappropriate precocious formation of astrocytes during the embryonic neurogenic period, and leads to a robust increase in the number of astrocytes generated. Second, as shown in the clonal experiments, SHP-2 mediates these effects by instructing neural precursors to generate neurons rather than astrocytes, thereby ensuring that precursors that are biased to a neuronal fate do not attempt to become glia even in the presence of cytokines. This occurs via two dissociable mechanisms; SHP-2 activates the neurogenic Ras-MEK-C/EBP pathway, and at the same time inhibits the gliogenic gp130-JAK-STAT pathway. Third, the constitutive activation of SHP-2 that occurs in Noonan Syndrome causes a large reduction in astrocyte formation, and a coincident increase in neuronal number in the forebrain and hippocampus. These effects are seen both following ectopic express of a Noonan Syndrome SHP-2 allele and, importantly, in a Noonan Syndrome mouse model carrying the same activated allele. Together these data indicate that SHP-2 regulates the timing and extent of neurogenesis versus gliogenesis by acting as a growth factor-regulated switch to bias cortical precursor cells to one fate and against another. Moreover, perturbations in the ratio of neurons versus glia following constitutive SHP-2 activation may contribute to the cognitive dysfunction observed in Noonan Syndrome individuals. The impact of this work will be further discussed in Chapter 5.
Figure 3.1
Figure 3.1: **SHP-2 expression is high in cortical precursors during the neurogenic, but not gliogenic period.** (a) Western blot analysis for SHP-2 in equal amounts of lysate from cortical precursors cultured for 1-5 DIV. (b) Immunocytochemical analysis for SHP-2 (red) and nestin (top panel, green) or βIII-tubulin (bottom panel, green) in cortical precursors cultured for 2 DIV. Arrows denote double-labelled cells. Space bar = 50μm. (c) Immunohistochemical analysis for SHP-2 (all panels, red) and nestin (left panels, green) or βIII-tubulin (right top panel, green) in coronal sections of the developing cortex at E12, E15, E17 and P5. Arrows denote nestin-positive precursors in the ventricular zone/subventricular zone (VZ/SVZ) that are double-labelled for SHP-2. The broken line demarcates the VZ/SVZ from the cortical mantle (CM). V = ventricle, CM = cortical mantle. Space bar = 50μm. Note that nestin-positive precursors in the VZ/SVZ express high relative levels of SHP-2 at E12 and E15, but not at E17 and P5. (d) Western blot analysis for SHP-2 in equal amounts of lysate from NIH 3T3 cells that were transfected with plasmids encoding either SHP-2 shRNA1 or a scrambled shRNA control (scr) for 48 or 72 hours. Blots were reprobed with an antibody to ERK to ensure equal protein loading. (e) Immunocytochemical analysis for SHP-2 (red) in transfected cortical precursors (green) indicating no or low (no-low), basal or high levels of SHP-2 expression. Each lower and upper set of panels shows the same field, and the arrows indicate the same transfected cell in both. Space bar = 25μm. (f) Quantitation of SHP-2 immunoreactivity as shown in (e) for cortical precursors cotransfected with plasmids encoding EGFP and shRNA1, a scrambled shRNA (scr) or D61G SHP-2 for 2 DIV.
Figure 3.2
**Figure 3.2:** *Genetic knockdown of SHP-2 has no effect on cortical precursor cell survival or proliferation, but specifically inhibits the genesis of neurons.* (a) Immunocytochemical analysis for EGFP (GFP; green) and βIII-tubulin (red) in cortical precursors cotransfected with plasmids encoding EGFP and SHP-2 shRNA1 or scrambled shRNA and cultured for 3 DIV. Cells were counterstained with Hoechst 33258 to show all of the nuclei in the field. Arrows denote double-labelled, transfected cells, and the arrowhead denotes an EGFP-positive cell that is not double-labelled. Space bar = 50 μm. (b) Quantitation of the percentage of transfected, βIII-tubulin-positive neurons in experiments similar to that shown in panel (a). Three representative experiments of 8 are shown. In experiment 3 (Exp 3), one additional set of sister cultures was cotransfected with plasmids encoding SHP-2 shRNA1 (which is specific to the murine sequence), and an HA-tagged wildtype form of human SHP-2 (hWT SHP-2). Error bars denote SEM. *p<0.05, **p<0.01, ANOVA. (c) Quantitation of cortical precursors transfected as in (a), cultured for 2 DIV, and immunostained for Ki67 to monitor cellular proliferation. Two representative experiments of three performed are shown. Error bars denote SEM. p>0.05, ANOVA. (d,e) Quantitation of cortical precursors transfected as in (a), cultured for 2 DIV, and then immunostained for cleaved caspase-3 to monitor apoptosis (d) and counterstained with Hoechst 33258 to monitor nuclear morphology (e). Two representative experiments of three performed are shown. Error bars denote SEM. p>0.05, ANOVA(d) and Student’s t-test (e). (f) Quantitation of cortical precursors transfected as in (a), cultured for 3 DIV in the presence of the pan-caspase inhibitor ZVAD, and immunostained for βIII tubulin to monitor neuronal differentiation. Two pooled experiments are shown. Error bars denote SEM. ***p<0.005, Student’s t-test.
Figure 3.3
Figure 3.3: *(a-e) Genetic knockdown of SHP-2 enhances cytokine-mediated gliogenesis.*

(a) Immunocytochemical analysis for EGFP (GFP; green) and the astrocyte marker GFAP (red) in cortical precursors cotransfected with plasmids encoding a nuclear EGFP and SHP-2 shRNA1, exposed to 50 ng/ml CNTF at 2 DIV, and cultured for 4 additional days. Cells were counterstained with Hoechst 33258 to show all of the nuclei in the field. Arrows denote double-labelled, transfected cells. Space bar = 50 µm. (b) Quantitation of the percentage of transfected, GFAP-positive astrocytes in experiments similar to that shown in panel (a). Two representative experiments of three are shown. Error bars denote SEM. *p<0.05, ANOVA. (c) Quantitation of the percentage of transfected, CD44-positive cells in an experiment similar to that described in (a). Error bars denote SEM. ***p<0.005, Student’s t-test. (d) Quantitative analysis for GFAP-immunoreactive astrocytes in cultures of cortical precursors cotransfected with plasmids encoding GFP and a dominant-negative form of SHP-2, ΔP-SHP-2, exposed to CNTF at 2 DIV, and immunostained after an additional 4 days. Three representative experiments are shown. Error bars denote the SEM. **p<0.01, ***p<0.005, Student’s t-test. (e) Quantitative analysis for βIII tubulin immunoreactive neurons in cultures of cortical precursors transfected with plasmids encoding GFP and ΔP-SHP-2, and immunostained after 3 DIV. Error bar denote SEM. p>0.05, Student’s t-test. *(f-l) Clonal analysis demonstrates that SHP-2 instructs precursor cells to generate neurons versus astrocytes.* (f) Triple-label immunocytochemistry for EGFP (GFP, green), the neuronal marker βIII tubulin (blue) and the astrocyte marker GFAP (red) in cortical precursors cotransfected with plasmids encoding a nuclear EGFP and SHP-2 shRNA, exposed to 50 ng/ml CNTF at 2 DIV and cultured for an additional 4 days. Arrows and arrowheads denote EGFP-positive astrocytes and neurons, respectively. Space bar = 50 µm. (g) Quantitation of three pooled experiments similar to that shown in (f) where precursors were exposed to CNTF 2 days following transfection, and were analyzed 2 days later (4 days in total). Error bars denote SEM. * p<0.05, **p<0.01, Student’s t-test. (h) Quantitation of two representative experiments of three similar to that shown in (f), where cortical precursors were cultured for 4 or 6 DIV in the absence of CNTF. (i) Triple-label immunocytochemistry of cortical precursors cotransfected with EGFP (green) and SHP-2 shRNA1 for 2 days, exposed to CNTF for 2 days, and then stained for βIII-tubulin (blue) and GFAP (red). Each panel shows the same transfected precursors that is coexpressing all three proteins (arrows).
Scale bar = 25μm. (j) Quantitation of 2 pooled experiments (of three performed) similar to that shown in (f), where cortical precursors were transfected and cultured for 2 days, CNTF was added for an additional 2 days (total of 4 days), and cells were in the presence of the pan-caspase inhibitor ZVAD from day 1 on. Cells were double-labelled for βIII-tubulin and EGFP. Error bars denote SEM. *p<0.05, Student's t-test. (k) Quantitation of the percentage of neurogenic and gliogenic clones in experiments similar to that shown in panel (f), where cells were cotransfected with plasmids encoding EGFP and empty vector or D61G SHP-2, showing pooled data from 4 independent experiments. Error bars denote SEM. * p<0.05, **p<0.01, Student’s t-test. (l) Quantitation of two representative experiments (of three performed) similar to that shown in (j) except that precursors were cotransfected with plasmids encoding EGFP and scrambled vector or D61G SHP-2.
Figure 3.4
Figure 3.4: A Noonan Syndrome activated SHP-2 mutant promotes neurogenesis and inhibits gliogenesis in cultured cortical precursors. (a) Immunocytochemical analysis for GFP or HA (green) and βIII-tubulin (red) in cortical precursors transfected with plasmids encoding a nuclear EGFP or HA-tagged D61G SHP-2 mutant, and cultured for 3 DIV. Cells were counterstained with Hoechst 33258 to show all of the nuclei in the field. Arrow denotes a double-labelled, transfected cell. (b) Quantitation of the percentage of transfected, βIII-tubulin-positive neurons in experiments similar to that shown in panel (a). Two experiments are shown. In both experiments, sister cultures were also transfected with plasmids encoding wildtype (WT) SHP-2. Error bars denote SEM. ** p<0.01, ANOVA. (c) Quantitation of cortical precursors transfected as in (a), cultured for 2 DIV, and immunostained for Ki67 to monitor cellular proliferation. Three pooled experiments are shown. Error bars denote SEM. **p<0.01, Student’s t-test. (d,e) Quantitation of cortical precursors transfected as in (a), cultured for 2 DIV, and then immunostained for EGFP and βIII-tubulin and counterstained with Hoechst 33258 to monitor nuclear morphology. Panel (d) shows the percentage of live cells in 2 independent experiments, while (e) shows the percentage of βIII-tubulin-positive and negative cells with normal (live) versus apoptotic (dead) nuclear morphology within an individual experiment. Error bars denote SEM. *p<0.05, **p<0.01, Student’s t-test. (f) Quantitation of cortical precursors transfected as in (a), cultured for 3 DIV in the presence of the pan-caspase inhibitor ZVAD, and immunostained for βIII tubulin to monitor neuronal differentiation. One representative experiment of two is shown. Error bars denote SEM. ** p<0.01, Student’s t-test. (g) Immunocytochemical analysis for HA (green) and GFAP (red) in cortical precursors transfected with plasmid encoding nuclear EGFP or the HA-tagged WT SHP-2 or D61G SHP-2 mutant, exposed to 50 ng/ml CNTF at 2 DIV, and cultured for 4 additional days. Cells were counterstained with Hoechst 33258 to show all of the nuclei in the field. Arrowheads denote a transfected cell that is not GFAP-positive. (h) Quantitation of the percentage of transfected, GFAP-positive astrocytes in experiments similar to that shown in panel (g). Two representative experiments of six are shown. Error bars denote SEM. *p<0.05, ** p<0.01, ANOVA.
Figure 3.5

(a) Scrambled and shRNA1 transfected cells that have migrated to CM. Percentage of transfected cells that have migrated to CM for scrambled and shRNA1 transfected cells.

(b) Graph showing the percentage of transfected cells that have migrated to CM for scrambled and shRNA1 transfected cells at 3 and 4 days.

(c) Graph showing the percentage of HuD+ve transfected cells for scrambled and shRNA1 transfected cells at 3 and 4 days.

(d) Graph showing the percentage of Ki67+ve transfected cells for scrambled and shRNA1 transfected cells at 3 and 4 days.

(e) Graph showing the percentage of Hoechst+ve transfected cells for scrambled and shRNA1 transfected cells at 3 and 4 days.

(f) Graph showing the percentage of GFP+ve transfected cells for scrambled and shRNA1 transfected cells at 3 and 4 days.

(g) Graph showing the percentage of Cleaved caspase-3+ve transfected cells for scrambled and shRNA1 transfected cells at 3 and 4 days.
**Figure 3.5. Genetic knockdown of SHP-2 inhibits cortical neurogenesis in vivo.** Precursor cells of the E13/E14 cortex were transfected by *in utero* electroporation with plasmids encoding a nuclear EGFP and with either SHP-2 shRNA1 or a scrambled shRNA, and were then analyzed four days later. (a) Immunocytochemical analysis for EGFP (GFP; green) in coronal sections of the telencephalon. Tissue sections were counterstained with Hoechst 33258 to show the overall morphology of the electroporated cortex. h = hippocampus, VZ = ventricular zone, SVZ = subventricular zone, CM = cortical mantle. Scale bar = 100µm. (b) Quantitation of the percentage of transfected, GFP-positive cells that migrated within the cortical mantle in experiments similar to that shown in (a). For 4 days, n = 6 each of control and experimental brains, with at least four adjacent sections analyzed for each brain. Error bars denote SEM. ***p<0.005 (Student’s t test). (c) Confocal image of immunohistochemical analysis for EGFP (GFP; green) and the neuronal marker HuD (red) in the cortical plate. Arrows denote examples of double-labelled, transfected cells. Scale bar = 20µm. (d) Quantitation of experiments similar to that in panel (c) showing the percentage of HuD-positive transfected cells present within the cortex of animals electroporated with plasmids encoding SHP-2 shRNA1 or a scrambled shRNA. For 4 days, n = 6 each of control and experimental brains, with at least four adjacent sections analyzed/brain. Error bars denote SEM, ***p<0.005, Student’s t-test. (e) Fluorescence micrographs of sections double-labelled for EGFP (GFP) and for cleaved caspase-3 (red) to monitor apoptosis. Sections were counterstained with Hoechst 33258 to show nuclei of all of the cells in the section. Arrow denotes a rare, double-labelled, transfected cell. Scale bar = 50µm. (f) Confocal micrographs of the VZ/SVZ of sections double-labelled for EGFP (GFP; green) and Ki67 (red) to monitor proliferation. Arrow indicates an example of colocalization. Scale bar = 20µm. (g) Quantitation of tissue sections similar to those shown in panel (f) showing the percentage of transfected, Ki67-positive cells in the ventricular/subventricular zone. Error bars denote SEM. p>0.05, Student’s t-test.
Figure 3.6
Figure 3.6. Inhibition of SHP-2 perturbs both the timing and magnitude of gliogenesis in vivo.

(a-d) Precursor cells of the E14 cortex were transfected by in utero electroporation with plasmids encoding a nuclear EGFP and with either SHP-2 shRNA1 or a scrambled shRNA, and were then analyzed at E17 (a), E18 (b), or P3 (c,d). (a) Immunohistochemical analysis for EGFP (GFP; green) and the astrocyte marker GFAP in a coronal section of the subventricular zone of an E17 brain transfected with SHP-2 shRNA1 at E14. The arrow denotes a double-labelled cell. Scale bar = 50μm. (b) Immunohistochemical analysis for EGFP (GFP; green) and GFAP (red) in coronal sections of the subventricular zone of E17/18 brains transfected at E14 with plasmids encoding a scrambled shRNA (left panel) and SHP-2 shRNA1 (right panels). Note the paucity of GFAP immunostaining in the scrambled shRNA-transfected section compared to the SHP-2 shRNA-transfected section. The arrow denotes a transfected, double-labelled cell. Scale bar = 50μm. (c) Confocal micrographs of the SVZ in sections of brains transfected at E14, and analyzed at P3 by immunocytochemistry for EGFP (GFP; green) and GFAP (red). Arrows indicate transfected, double-labelled cells. Scale bar = 20μm. (d) Quantitation of the percentage of transfected, GFAP-positive cells within the subventricular zone in experiments similar to that shown in (c). n = 3 brains each in control and experimental groups. (e,f) Quantitation of experiments similar to those shown in panels (c,d), where embryos were transfected at E14 with plasmids encoding EGFP and either the ΔP-SHP-2 mutant or the empty vector, and analyzed immunocytochemically for EGFP and GFAP (e) or CD44 (f) at P3. The data are expressed as the percentage of transfected, GFAP-positive cells within the SVZ. In (e), n = 7 brains each in control and experimental groups, with at least four adjacent sections analyzed per brain. Error bars denote SEM. **p<0.01, ***p<0.001, Student’s t-test. (g) Quantitation of experiments similar to those shown in (e,f) except that brains were analyzed 4 days after electroporation by immunostaining for EGFP and HuD. n = 2 each of control and experimental brains, with at least four adjacent sections analyzed per brain. Error bars denote SEM. p>0.05, Student’s t-test. (h) Coronal sections of P3 cortex from brains electroporated at E14 with plasmids encoding a scrambled, control shRNA, or SHP-2 shRNA1, and then analyzed at P3. Sections were immunolabelled for EGFP (GFP; green) and counterstained with Hoechst 33258 to show the overall histology of the electroporated
area. SVZ = subventricular zone, CL= cortical layers. Scale bar = 100µm. (i) Quantitation of sections similar to those shown in panel (h) to ascertain the percentage of transfected, GFP-positive cells that were present anywhere within the cortical layers (total) or that were present in layers II and III (total layers II & III). n = 3 each for control and experimental groups, with at least four equivalent adjacent sections analyzed/brain. Error bars denote SEM. ***p<0.001, Student’s t-test. (j) Confocal micrograph of a section similar to those in (h) immunostained for EGFP (green) and HuD (red). The image shows both the SVZ and the deeper cortical layers (CL) adjacent to the SVZ. Arrows denote transfected, double-labelled neurons. Scale bar = 20µm. (k) Quantitation of the percentage of transfected cells within the cortical layers that were HuD-positive. n = 3 each of experimental and control brains. Error bars denote SEM. p>0.05, Student’s t test.
Figure 3.7
Figure 3.7: A Noonan Syndrome-associated activated SHP-2 mutant enhances neurogenesis and inhibits gliogenesis in vivo. Precursor cells of the E13/14 cortex were transfected by in utero electroporation with plasmids encoding a nuclear EGFP and a constitutively activated human Noonan Syndrome SHP-2 mutant (D61G SHP-2), wildtype SHP-2 (WT) or the empty vector, and then were analyzed at E17/18 (a,b) or at P3 (c-g). In panel (b), plasmids were electroporated into the brains of E13/14 Tα1:nlacZ mice. (a) Quantitation of the percentage of transfected, HuD-positive neurons in coronal sections of the E17/18 cortex double-labelled for EGFP (GFP) and HuD. n = 3 control and 4 experimental brains. Error bars denote SEM. *p<0.05, Student’s t-test. (b) Quantitation of the percentage of transfected cells that expressed the Tα1:nlacZ transgene 3 days following electroporation, as detected by double-label immunocytochemistry for EGFP and β-galactosidase. n = 2 control and 2 experimental brains. Error bars denote SEM. ***p<0.005, Student's t-test. (c,d) Confocal micrographs of coronal sections through the SVZ of P3 brains double-labelled for EGFP (GFP; green) and GFAP (c, red) or CD44 (d, red). Arrows denote double-labelled cells. Scale bar = 20μm. (e-g) Quantitation of the percentage of transfected, GFAP-positive (e,f) or CD44-positive (g) cells within the SVZ in experiments similar to those shown in panel (c). In (e), n = 7 experimental and 6 control brains, in (f), n = 4 experimental and 5 control brains, and in (g), n=2 each of experimental and control brains. In all cases, at least 4 equivalent, adjacent sections were analyzed per brain. Error bars denote SEM. *p<0.05, **p<0.01, Student's t-test.
Figure 3.8
Figure 3.8: (a-c) A Noonan syndrome-associated SHP-2 mutant enhances neurogenesis via MEK. (a) Immunocytochemical analysis for EGFP (GFP; green) and activated, phosphorylated ERK (phosphoERK; red) in cortical precursor cells cotransfected with plasmids encoding EGFP (green) and the constitutively-activated human Noonan Syndrome mutant, D61G SHP-2 and cultured for 3 DIV. Arrows denote transfected cells that express high levels of phosphoERK, while the dotted arrow indicates a transfected cell expressing basal levels of phosphoERK. Scale bar = 25μm. (b) Quantitation of transfected, phosphoERK-positive cells in one representative experiment of two where precursors were transfected with EGFP plus D61G SHP-2 or SHP-2 shRNA1. PhosphoERK expression was quantitated relative to basal levels as shown in (a). Error bars denote SEM. *p<0.05, ANOVA. (c) Quantitation of the percentage of βIII-tubulin-positive, transfected cells in three independent experiments where precursors were transfected with plasmids encoding EGFP or HA-tagged D61G SHP-2, treated with a pharmacological MEK inhibitor PD98059 after 1 DIV, and analyzed 2 days later. Error bars denote SEM. *p<0.05, **p<0.01, ANOVA. (d-g) SHP-2 inhibits gliogenesis by negatively regulating gp130-JAK-STAT signaling. (d) Immunocytochemical analysis for EGFP (GFP; green) and phosphoSTAT-3 (red) in precursors cotransfected with plasmids encoding EGFP and SHP-2 shRNA1, treated with 50ng/ml CNTF at 2 DIV and cultured for one additional day. Cultures were counterstained with Hoechst 33258 to show all of the nuclei in the field. The arrow indicates a double-labelled cell. Scale bar = 50μm. (e) Quantitation of the percentage of transfected, phosphoSTAT-3-positive cells in one representative experiment of 3 performed that were similar to that shown in (d). Cultures were cotransfected with plasmids encoding EGFP and SHP-2 shRNA1, scrambled shRNA, the ΔP-SHP-2 mutant, or the activated, Noonan Syndrome D61G SHP-2 mutant. Error bars denote SEM. *p<0.05, ANOVA. (f) Quantitation of the percentage of transfected, GFAP-positive cells in two independent experiments where precursors were cotransfected with plasmids encoding EGFP and SHP-2 shRNA1 or scrambled shRNA, and then treated at 2 DIV with 50ng/ml CNTF with or without the pharmacological JAK2 inhibitor AG490 for an additional 4 days prior to analysis by double-labelling for EGFP and GFAP. DMSO was used as a control for the drug treatment. Error bars denote SEM. *p<0.05,**p<0.01, ANOVA.
Figure 3.9
Figure 3.9: A Noonan Syndrome mouse model shows enhanced neurogenesis and decreased gliogenesis in the neonatal hippocampus and cortex. (a) Micrograph of a coronal section through the neonatal forebrain, depicting the two regions that were analyzed, one in the hippocampus (region 1) and one in the dorsal cortex (region 2). (b-e) Analysis of the postnatal day 0 (P0) dentate gyrus (region 1). Sections through the extent of the dentate gyrus were immunostained for either GFAP or HuD, and then counterstained with Hoechst 33258 to show all of the nuclei in the analyzed region. (b) Immunohistochemical analysis for the astrocytic marker GFAP (red, bottom panels) in coronal sections at the same rostral-caudal level of the P0 Noonan syndrome (D61G/+) or wildtype (+/+), brain. Cell density was determined by the Hoechst stain (white in top panels and blue in bottom panels). Dashed outline represents the area that was analyzed. Arrows denote GFAP-positive cells. Scale bar = 50\(\mu\)m (top panels) and 25\(\mu\)m (bottom panels). (c) Quantitation of mean total cell density in the dentate gyrus in sections similar to those shown in panel (b), as assessed by counting Hoechst-positive nuclei. n=6 and 5 in the control and Noonan syndrome groups, respectively, with at least three adjacent sections analyzed per brain. Error bars denote SEM. *p<0.05, Student's t-test. (d) Quantitation of mean HuD-positive neuronal density (left panel) and the percentage of HuD-positive cells/total cells (right panel) in the dentate gyrus of sections similar to those shown in (b). n=6 and 5 in the control and Noonan syndrome groups, respectively, with at least three adjacent sections analyzed per brain. Error bars denote SEM. *p<0.05, ***p<0.005, Student's t-test. (e) Quantitation of mean GFAP-positive cell density (left panel) and the percentage of GFAP-positive cells/total cells (right panel) in the dentate gyrus of sections similar to those shown in (b). n=6 each in the control and Noonan syndrome groups, respectively, with at least three adjacent sections analyzed per brain. Error bars denote SEM. ***p<0.005, Student's t-test. (f-k) Analysis of the postnatal day 2 (P2) dorsal cortex (region 2). Sections through the dorsal cortex at this level were immunostained for either GFAP or NeuN, and then counterstained with Hoechst 33258 to show all of the nuclei in the analyzed region. (f) Immunohistochemical analysis for the neuronal marker NeuN (green, right panels) in coronal sections at the same rostral-caudal level of the P2 Noonan syndrome (D61G/+) or wildtype (+/+) brain. Cell density was determined by the Hoechst stain (white in left panels). SVZ = subventricular zone, CP = cortical plate. Scale bar = 100\(\mu\)m. (g,h) Immunocytochemical analysis for GFAP (red) in the
subventricular zone (SVZ) (g) or the glia limitans (h) at the same level of the dorsal cortex of P2 Noonan syndrome (D61G+/+) versus wildtype (+/+) brains. Arrows denote GFAP-positive cells. Scale bar = 50μm. (i) Quantitation of mean total cell density in the dorsal cortex in sections similar to those shown in panel (f), as assessed by counting Hoechst-positive nuclei. n=2 in each of control and Noonan syndrome groups, respectively, with at least four adjacent sections analyzed per brain. Error bars denote SEM. *p<0.05, Student’s t-test. (j) Quantitation of mean NeuN-positive neuronal density (left panel) and the percentage of NeuN-positive cells/total cells (right panel) in the dorsal cortex of sections similar to those shown in (f). n=2 in each of the control and Noonan syndrome groups, respectively, with at least four adjacent sections analyzed per brain. *p<0.05, ***p<0.005, Student's t-test. (j) Quantitation of mean GFAP-positive cell density (left panel) and the percentage of GFAP-positive cells/total cells (right panel) in the subventricular zone of the dorsal cortex in sections similar to those shown in (g). n=3 for controls, and 2 for Noonan syndrome brains, with at least four adjacent sections analyzed per brain. Error bars denote SEM. ***p<0.005, Student's t-test.
Chapter 4: Lfc and Tctex-1 regulate neurogenesis from radial cortical precursors

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“Lfc and Tctex-1 regulate neurogenesis from radial cortical precursors”

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Data shown in Figure 1, panels a,d,f; Figure 5a; Figure 8a,c were generated by D.C.L, with A.G.F.’s assistance with early cortical tissue dissections, precursor cultures and transfections, in utero electroporation, tissue preparation and mitotic spindle orientation quantification. A.G.F. did not participate in experiments involved in generating Figure 1 panel e, an important control for our experiments done by D.C.L. The remainder of the data and figures, with the assistance of D.C.L. with plasmid DNA preparations and intellectual contributions were generated by A.G.S. M.G from R.R.’s lab generated all of the shRNA constructs used in the experiments and provided the anti-Lfc antibodies. R.R. and D.R.K. contributed intellectually, and F.D.M. contributed intellectually and to the writing of the manuscript.

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4.1 SUMMARY
The mechanisms that regulate symmetric, self-renewal versus asymmetric, neurogenic divisions of mammalian neural precursors are still not well-understood. Here, we demonstrate that Lfc, a Rho-GEF that interacts with spindle microtubules, and its negative regulator Tctex-1, together determine the genesis of neurons from radial precursors in the embryonic murine cortex. Specifically, genetic knockdown of Lfc in cortical precursors either in culture or in vivo inhibits neurogenesis and instead maintains cells as cycling radial precursors. Conversely, genetic knockdown of Tctex-1 in radial precursors promotes neurogenesis and depletes cycling cortical precursors. Coincident silencing of these two genes indicates that Tctex-1 normally inhibits the genesis of neurons from radial precursors by antagonizing the proneurogenic actions of Lfc. Moreover, Lfc is required to determine the orientation of mitotic precursor cell divisions in vivo. Thus, Lfc and Tctex-1 interact to regulate cortical neurogenesis, potentially by regulating mitotic spindle orientation.

4.2 INTRODUCTION AND RATIONALE
During development of the mammalian neocortex, the genesis of neurons and glia from neural precursors occurs in a precisely timed fashion. Molecular mechanisms have evolved to control neural precursor expansion prior to neurogenesis, and to allow for an exponential generation of neurons while ensuring maintenance of sufficient precursors to produce later-born neurons, glial cells and adult neural stem cells (reviewed in Götz & Huttner, 2005; Zhong & Chia 2008). A heterogeneous population of precursors, dividing either symmetrically to generate cells of similar fate or asymmetrically to generate cells of different fates, plays roles at each of these steps. Of these, radial precursors generate the majority of neurons directly or indirectly. Radial precursors arise from neuroepithelial stem cells in the ventricular zone (VZ) and at early stages divide symmetrically, while they divide asymmetrically during neurogenesis to self-renew and at the same time generate either a
neuron or an intermediate/basal precursor. Basal precursors generally do not self-renew, and divide in the subventricular zone (SVZ) to generate two neurons (Miyata et al., 2004; Noctor et al., 2004; Haubensak et al., 2004; Gal et al., 2006). At the end of neurogenesis, some radial precursors undergo a terminal symmetric division to yield two neurons (Haydar et al., 2003). While most remaining radial precursors generate astrocytes once neurogenesis is complete, some persist in the adult forebrain SVZ as adult neural stem cells (Bonfanti & Peretto, 2007).

Both the timing and extent of neurogenesis during cortical development are controlled by many extrinsic signals and intracellular biases (reviewed in Chapter 1). However, the mechanisms that regulate the switch from symmetric to asymmetric precursor cell division during neurogenesis are less clear. One proposed mechanism is the regulation of spindle orientation and cleavage plane in dividing radial precursors, which changes over the course of cortical development and correlates with the cell-fate decisions of daughter cells (Chenn & McConnell, 1995; Haydar et al., 2003). Although much work has been done in model systems such as Drosophila melanogaster and Caenorhabditis elegans (reviewed in Gönczy, 2008; Knoblich, 2008) to support such a spindle orientation model, only a few polarity genes have been shown to control vertebrate precursor spindle orientation during neurogenesis, including LGN (Morin et al., 2007; Konno et al., 2008), Nde1 (Feng et al., 2004) LIS1 (Tsai et al., 2005; Yingling et al., 2008; Pawlisz et al., 2008), Par proteins (Costa et al., 2007) and signaling proteins such as AGS3 and G proteins (Sanada & Tsai, 2005), and doublecortin-like kinase (Shu et al., 2006).

In this regard, the guanine nucleotide exchange factor (GEF) Lfc has been shown to regulate spindle orientation in nonneural cells (Benais-Pont et al., 2003; Bakal et al., 2005), making it an interesting candidate to investigate within the context of cortical development. Lfc and its human orthologue, GEF-H1, are Dbl-family GEFs specific for Rho (Glaven et al., 1996; Ren et al., 1998; Glaven et al., 1999) and are unique among GEFs in their interaction with microtubules (Krendel et al., 2002). Interestingly, Lfc interacts with, and is negatively regulated by Tctex-1 (Greeve et al., submitted), a protein that is expressed in adult neural stem cells (Dedesma et al., 2006). While most extensively characterized as a light chain component of the dynein motor complex (King et al., 1996), Tctex-1 also has several dynein-independent functions including roles in neuronal growth (Chuang et al., 2005; Sachdev et
al., 2007), and G protein signaling activation (Takesono et al., 1999, Sachdev et al., 2007). Here, we have tested the idea that these two proteins might be important in embryonic neural precursors, and demonstrate that Lfc and Tctex-1 are part of an essential pathway that regulates the genesis of neurons from radial precursor cells, potentially by regulating mitotic spindle orientation.

**4.3 RESULTS**

**4.3.1 The Rho-GEF Lfc is essential for genesis of neurons from cortical radial precursors**

To evaluate the role of Lfc during cortical development, we first confirmed that it is expressed in the embryonic cortex, as previously-reported (Yoshizawa et al., 2003; Ryan et al., 2005). Western blot analysis of cortical lysates with anti-Lfc revealed that Lfc was expressed at the earliest time-point analyzed, embryonic day 11 (E11), when the cortical neuroepithelium is largely composed of neural precursors, and that expression persisted until at least postnatal day 2 (P2) (Fig. 1a). We then turned to primary cultures of E12 cortical precursors to define the cell types that expressed Lfc. We have shown previously that, upon plating, almost all E12 cortical cells are dividing, nestin-positive precursors, many of which then exit the cell cycle to become neurons over the ensuing 1 to 5 days in culture (Barnabé-Heider and Miller, 2003; Paquin et al., 2005; Barnabe-Heider et al., 2005; Gauthier et al., 2007). To further characterize these precursors, we immunostained them for two markers of radial precursors, BLBP and Pax6 (Feng et al., 1994; Götz et al., 1998). This analysis revealed that, upon plating, the vast majority (>95%) of the cells were BLBP-positive radial precursors (Fig. 1b). Three days later, double-label immunocytochemistry demonstrated that approximately 26% of the cells in the cultures were βIII-tubulin-positive neurons, while approximately 70% were proliferating, Ki67-positive cells, presumably precursors (Fig. 1c). Of these, almost all were BLBP-positive (approximately 68%), indicating that even at this later timepoint, the vast majority of precursors in these cultures were radial precursors.

To ask whether Lfc was expressed in these radial precursors, we performed immunocytochemistry eight hours after plating; this analysis revealed that Lfc was expressed in almost all of the nestin-positive and/or BLBP-positive precursors (Fig. 1d). To examine whether Lfc was also expressed in newly-born neurons, cultures were analyzed after three
days in vitro. Immunostaining for the neuronal marker βIII-tubulin revealed expression of Lfc in neurons (Fig. 1d), where it was localized to both cell bodies and neurites, as previously reported (Yoshizawa et al., 2003; Ryan et al., 2005).

To examine the function of Lfc in cortical precursors, we reduced endogenous levels of the protein using shRNA constructs that co-express GFP to mark transfected cells. Western blot analysis of lysates from 3T3 cells transfected with either one of two Lfc shRNAs targeted to two different sequences (Lfc shRNA #1 and #2) (Greeve et al., submitted) showed shRNA-mediated knockdown of Lfc protein levels (Fig. 1e). To confirm the efficacy of the shRNAs in cultured cortical precursors, cells were analyzed three days post-transfection by immunofluorescence staining to detect endogenous Lfc. While Lfc protein levels were unaffected in cells transfected with the scrambled shRNA control, Lfc shRNA-transfected cells expressed relatively little detectable Lfc protein in comparison to untransfected precursors in the same cultures (Fig. 1f).

We then used these shRNAs to ask whether Lfc was important for cortical precursors to generate neurons by transfecting E12 precursors at the time of plating and analyzing the culture 3 days later. We first confirmed that Lfc was not essential for cell survival. Analysis of nuclear morphology demonstrated that knockdown of Lfc with either shRNA had no effect on levels of apoptosis in transfected precursors (Fig. 1g). We then quantified neurogenesis; double-labelling for GFP and βIII-tubulin, indicated that transfection with either Lfc shRNA resulted in a nearly two-fold decrease in the number of neurons generated relative to the scrambled shRNA control (Fig. 1h). Thus, Lfc is essential for cultured radial precursors to generate appropriate numbers of neurons.

To ask whether Lfc is also essential for neurogenesis in vivo, we performed in utero electroporation into the E13.5 cortex using the same Lfc shRNAs. Immuno-cytochemical analysis of coronal sections from brains 4 days post-electroporation demonstrated that Lfc knockdown caused a decrease in the migration of GFP-positive cells from the VZ/SVZ to the cortical mantle (Fig. 2a). Quantification of the percentage of GFP-positive cells present within the cortical mantle demonstrated a decrease of approximately two-fold in cortices electroporated with Lfc shRNA #1 or #2 versus scrambled control shRNA (Fig. 2b). Instead, Lfc shRNA-transfected cells accumulated specifically within the SVZ (Fig. 2c), suggesting that Lfc-knockdown inhibited either the genesis and/or migration of newly-born neurons.
distinguish between these possibilities, we performed immunocytochemistry for GFP and the neuronal marker HuD (Fig. 2d). Quantification of the percentage of total GFP and HuD positive cells demonstrated that Lfc knockdown robustly decreased the percentage of transfected neurons generated in the embryonic cortex (Fig. 2e). Moreover, even the percentage of HuD-positive transfected cells within the VZ/SVZ was reduced (Fig. 2f), indicating that Lfc is required for the generation of neurons from precursor cells, and not for their migration out of the VZ/SVZ. This conclusion was confirmed by immunostaining for the neuronal precursor/neuron marker doublecortin and the early neuronal marker βIII-tubulin (Fig. 2g-i).

Analysis of electroporated cortices at P3 revealed that the perturbations induced by knockdown of Lfc persisted into the postnatal period. Specifically, the percentage of Lfc shRNA-transfected cells within the cortical mantle was decreased while the percentage of cells in the SVZ was robustly increased (Fig. 3a,b). Moreover, the percentage of total HuD-positive neurons was decreased when Lfc levels were reduced (Fig. 3c,d). Thus, Lfc normally functions to promote the genesis of neurons from cortical precursor cells and when its levels are reduced, the cells accumulate in the SVZ.

### 4.3.2 Reducing Lfc levels maintains cells as cycling radial precursors

One way that Lfc knockdown might inhibit neurogenesis is by maintaining cells as cycling precursors, while a second explanation is that precursor fate might be subverted to the glial lineage. To distinguish these possibilities, we knocked-down Lfc levels in cultured radial precursors, and asked if this perturbed either the number of proliferating precursors or the generation of astrocytes. We assessed the effects on proliferation by measuring the percentage of transfected cells that were positive for Ki67, which is expressed in late G1, S, G2 and M phases of the cell cycle. This analysis revealed that the percentage of proliferating cells two days following transfection was significantly increased when Lfc levels were reduced with either of the two shRNAs (Fig. 4a). To confirm this effect, we performed clonal analysis; cultured radial precursors were transfected at very low density with Lfc or scrambled shRNAs, and the number of GFP-positive progeny of individual transfected precursors determined 3 days later. This analysis demonstrated that Lfc knockdown resulted in fewer single cell clones, and more clones of greater than 5 cells (Fig. 4b),
consistent with increased proliferation. Next, we asked whether reduced Lfc levels affected the endogenous astrogenesis that occurs in these cortical precursor cultures by 7 days. Immunostaining for the astrocyte marker GFAP showed that similar numbers of astrocytes were generated whether or not Lfc levels were decreased (Fig. 4c,d). Thus, Lfc is required for neurogenesis but not astrogenesis, and precursors in which Lfc is reduced continue to cycle.

To ask if Lfc plays the same role in vivo, we performed in utero electroporation with Lfc shRNAs and analyzed proliferation in the cortex 2 days later. Immunostaining for Ki67 demonstrated that the number of cycling, Ki67-positive precursors was increased with Lfc knockdown (Fig. 4e,f). To confirm this phenotype, we monitored cell cycle status by injecting pregnant mothers with the DNA nucleotide analogue BrdU at E14.5, one day after electroporation with either Lfc or scrambled shRNA. Analysis for Ki67 and BrdU incorporation 24 h following injection demonstrated that both Lfc and scrambled shRNA-transfected cells incorporated BrdU equally (p>0.05 in each of 2 independent experiments, n = 7 brains for each treatment), but that the percentage of BrdU-positive cells that were also Ki67-positive was significantly increased with Lfc shRNA (Fig. 4e,g). Thus, the percentage of precursors maintained in the cell cycle over the timeframe of the experiment was increased by Lfc knockdown.

To determine whether a specific class of cortical precursors was affected, electroporated cortices were immunostained at 2 days following electroporation for Pax6, a marker for radial precursors, or for Tbr2, a marker for basal progenitors. Consistent with the culture data, decreased Lfc levels resulted in more Pax6-positive cells (Fig. 4h,i). Interestingly, while the morphology of these Lfc shRNA-transfected radial precursors was not apparently changed (Fig. 4j), a greater percentage of them were localized to the SVZ (Fig. 4k), consistent with the observed accumulation of cells within the SVZ (Fig. 2c, 3b). In contrast, the percentage of Tbr2-positive basal progenitors was decreased (Fig. 4l,m). Since one way radial precursors can generate neurons is via Tbr2-positive basal progenitors (Haubensak et al, 2004; Noctor et al, 2004), then these data suggest that Lfc normally functions to promote the radial precursor to basal progenitor transition.
4.3.3 The Lfc negative regulator, Tctex-1, functions to inhibit neurogenesis

One protein that binds to and negatively regulates Lfc is the dynein light chain component Tctex-1 (Greeve et al., submitted). Since Tctex-1 has been described as a marker for neural precursors (Dedesma et al., 2006), we asked whether it might function to maintain the precursor state by inhibiting Lfc-mediated neurogenesis. To do this, we initially confirmed that Tctex-1 was expressed during embryonic cortical development, as previously reported (Chuang et al., 2001; Sachdev et al., 2007). Western blot analysis with anti-Tctex-1 demonstrated that it was expressed in the embryonic cortex from E12, when the neuroepithelium is almost completely composed of precursors, to at least E18 (Fig. 5a). We then asked whether Tctex-1 regulated neurogenesis by transf ecting cultured cortical precursors with two previously-characterized Tctex-1 shRNAs that silence Tctex-1 expression in neurons and nonneural cells (Chuang et al., 2005; Greeve et al, submitted). Double label analysis for GFP and βIII-tubulin revealed that knockdown of Tctex-1 resulted in a significantly increased number of neurons relative to scrambled shRNA controls (Fig. 5b). Thus, in contrast to Lfc, Tctex-1 functions to inhibit neurogenesis from radial precursors.

To ask if Tctex-1 also negatively regulates neurogenesis in vivo, we analyzed coronal sections of cortices electroporated in utero with Tctex-1 shRNAs at E13.5. At 4 days post-electroporation, reducing Tctex-1 levels resulted in an increased proportion of transfected cells within the cortical mantle (Fig. 5c,d). Immunostaining with the neuronal marker HuD confirmed that the majority of the cells within the cortical mantle were neurons and that a greater percentage of total Tctex-1 shRNA-transfected cells were neurons (Fig. 5c,e). Moreover, relative to controls, approximately 2.5 times more of the Tctex-1-transfected cells within the VZ/SVZ expressed a neuronal phenotype (Fig. 5f). Thus, Tctex-1 normally functions to inhibit cortical neurogenesis.

Analysis of similarly-electroporated cortices at post-natal day 3 revealed that these changes persisted into neonatal life (Fig. 5g); a greater percentage of total Tctex-1 shRNA transfected cells were HuD-positive neurons (Fig. 5h), with as many as 20% of the Tctex-1 shRNA-transfected cells within the VZ/SVZ expressing neuronal markers (Fig. 5i). In addition, of those Tctex-1 shRNA-transfected neurons that were present within the cortical
mantle, many were mislocalized. In control electroporated brains, virtually all of the transfected neurons were in layers II & III, as we have previously published (Paquin et al., 2005; Gauthier et al., 2007), while in the brains electroporated with Tctex-1 shRNA, many neurons were localized to layers IV to VI (Fig. 5g,j). Thus, knockdown of Tctex-1 resulted in enhanced neurogenesis, and in aberrant migration of many of the neurons that were generated.

If Tctex-1 inhibits neurogenesis by negatively regulating Lfc, then one would predict that the enhanced neurogenesis seen with Tctex-1 knockdown would occur at the expense of cycling precursors. To test this prediction, we electroporated embryos with Tctex-1 versus scrambled shRNAs at E13.5, and analyzed the cortices 2 days later. Immunostaining for Ki67 revealed that Tctex-1 knockdown decreased the percentage of proliferating cells within the VZ/SVZ (Fig. 6a,b). Furthermore, immunostaining for Pax6 demonstrated a decrease in the percentage of transfected radial precursor cells when Tctex-1 was knocked-down (Fig. 6c,d), although the morphology of the transfected radial precursors was apparently normal (Fig. 6e). Thus, Tctex-1 normally functions to inhibit neurogenesis and to maintain cycling radial precursors.

4.3.4 Tctex-1 inhibits cortical neurogenesis by antagonizing the actions of Lfc

Since our data show that Lfc and Tctex-1 have opposing functions during neurogenesis and since Tctex-1 is a known negative regulator of Lfc (Greeve et al., submitted), we asked whether Tctex-1 inhibits neurogenesis via Lfc. Specifically, we co-electroporated E13 cortices in utero with plasmids encoding Lfc shRNA plus dsRed and Tctex-1 shRNA plus EGFP to determine if coincident reduction of Lfc levels would rescue the Tctex-1 knockdown phenotype. As controls, we electroporated cortices with the Lfc or Tctex-1 shRNA plasmids and a plasmid encoding a scrambled shRNA plus either EGFP or dsRed. Cells expressing EGFP, dsRed and HuD were then quantified after immunocytochemistry for GFP and HuD (Fig. 7a). This analysis showed that, as predicted, co-electroporation of Tctex-1 and scrambled shRNAs caused an increase in the number of cotransfected cells in the cortical mantle (Fig. 7b), an increase in the total number of HuD-positive neurons (Fig. 7a,c), and a 4-fold increase in the number of neurons within the VZ/SVZ (Fig. 7d). However, coincident knockdown of Lfc with Lfc shRNA #1 completely
rescued these phenotypes (Fig. 7b-d). Thus, Tctex-1 inhibits the genesis of neurons from cortical radial precursors by antagonizing the actions of Lfc.

### 4.3.5 Lfc regulates mitotic spindle orientation in the embryonic cortex

Since Lfc is associated with the mitotic spindle in some cell types (Benais-Pont et al., 2003), and since mitotic spindle orientation is thought to play a role in regulating neurogenesis (Zhong & Chia, 2008), we asked whether Lfc might affect cortical neurogenesis by regulating spindle orientation. To do this, we first asked whether Lfc was associated with the spindle in cortical precursors; cultured precursors were immunostained for Lfc and α-tubulin to monitor microtubules, and counterstained with Hoechst to visualize DNA. This analysis (Fig. 8a) showed that, as in cell lines, Lfc was associated with the mitotic spindle during precursor cell division. We then asked whether Lfc might regulate spindle orientation during precursor division in vivo. Cortices were electroporated with Lfc shRNA versus scrambled shRNA, and then, 2 days later, coronal sections were immunostained for GFP to monitor transfected cells, γ-tubulin to visualize the centrosomes, phospho-histone H3 to monitor the dividing chromosomes, and counterstained with Hoechst to visualize DNA (Fig. 8b). We then measured the angle between the ventricular surface and the line between the centrosomes of transfected, mitotic cells in metaphase, anaphase and telophase. An angle between 0-15 degrees from the plane of the ventricular surface was scored as a vertical division. This analysis revealed that knockdown of Lfc increased the number of cell divisions at the apical surface that had a vertical, potentially symmetric, plane of cell division (Fig. 8c). Thus, Lfc normally associates with the mitotic spindle and regulates its orientation in dividing cortical precursors, thereby potentially promoting neurogenesis.

### 4.4 CONCLUSIONS

The data presented here support four major conclusions. First, our data demonstrate that Lfc is essential for the normal genesis of neurons from embryonic radial precursors. Lfc knockdown in precursors in culture or in vivo blocks neurogenesis and maintains cells as cycling radial precursors. In contrast, it has no effect on astrocyte formation. Secondly, we show that a negative regulator of Lfc, Tctex-1, functions in an opposite manner, and
normally serves to inhibit neurogenesis. Genetic silencing of Tctex1 either in culture or in vivo increases neurogenesis and decreases the proportion of cycling radial precursors. Third, our data indicate that Lfc and Tctex-1 are part of a common genetic pathway, where Tctex-1 normally functions by inhibiting the proneurogenic effects of Lfc. Finally, we show that Lfc regulates mitotic spindle orientation in dividing apical precursors, thereby providing a mechanism to explain its ability to regulate neurogenesis. Specifically, Lfc knockdown enhances vertical, potentially symmetrical apical divisions, thereby increasing the proportion of dividing radial precursors, and decreasing the proportion of neurogenic basal progenitors and newly-born neurons. Together, our findings support a model where the number of asymmetric, neurogenic versus symmetric, self-renewing radial precursor divisions is regulated by the interplay between the microtubule-interacting Lfc, and its negative regulator Tctex-1. The impact of this work will be further discussed in Chapter 5.
Figure 4.1

(a) Western blot analysis showing expression levels of Lfc and ERK1/2 at different stages (E11, E11.5, E12, E15, E18, P2) in development.

(b) Immunohistochemistry images showing expression of βIII tubulin and nestin at 8 hours.

(c) Immunohistochemistry images showing expression of βIII tubulin and nestin at 72 hours.

(d) Immunofluorescence images showing expression of Lfc and βIII tubulin at 8 and 72 hours.

(e) Western blot analysis showing expression of Lfc shRNA #1 and Scr shRNA at different stages in development.

(f) Immunofluorescence images showing expression of GFP and Lfc with Scr shRNA and Lfc shRNA #1.

(g) Graph showing percentage of live cells and βIII tubulin+ve cells under different conditions.

(h) Graph showing percentage of live cells and βIII tubulin+ve cells under different conditions in different experiments.

(Dan Lin)
Figure 4.1: (a-d) *Lfc is expressed in radial precursors and early-born neurons during cortical development.* (a) Western blot for Lfc in cortical lysates from embryonic day 11 (E11) to postnatal day 2 (P2). The blot was reprobed for Erk1/2 as a loading control. (b) Immunocytochemical analysis for BLBP (red) and nestin (green) in cortical precursors 8 hours following plating from the E12.5 cortex. Cells were counterstained with Hoechst 33258 to show cell nuclei. Arrows indicate a double-labeled cell. Scale bar = 50 μm. (c) Immunocytochemical analysis for βIII-tubulin (green) and Ki67 (red) in cortical precursors cultured for 3 days. Scale bar = 50 μm. (d) Immunocytochemical analysis for Lfc (red) and either nestin (green, top right panel) or βIII-tubulin (green, bottom right panel) in cortical precursors cultured for 8 or 72 hours, as indicated. Scale bar = 10 μm. (e-h) *Genetic knockdown of Lfc inhibits neurogenesis in cultured precursors.* (e) Western blot for Lfc in lysates of NIH 3T3 cells transfected with Lfc shRNA#1 or #2, or a scrambled shRNA control (Scr) for 72 hours. Blots were reprobed for Erk1/2 to ensure equal protein loading. (f) Immunocytochemistry for Lfc (red) and GFP (green) in cultured precursors transfected with Lfc shRNA#1 or scrambled shRNA for 72 hours. Arrows indicate the same transfected cell in the left and right panels of each pair. Scale bar = 10 μm. (g) Quantification of the survival of cortical precursors 2 days following transfection with Lfc shRNA#1 versus scrambled (Scr) shRNA, as determined by the morphology of Hoechst-stained nuclei in transfected cells. Results are pooled from 3 independent experiments. (h) Quantification of transfected, βIII-tubulin-positive neurons in precursor cultures cotransfected with plasmids encoding EGFP and Lfc shRNA#1, Lfc shRNA#2 or scrambled shRNA (Scr) for 3 days. Three independent experiments are shown. *p<0.05, **p<0.01, student's t-test or ANOVA.
Figure 4.2

ba

% migration to CM

0

20

40

60

10

50

30

% migration

CM VZ

SVZ

0

20

40

60

10

50

30

% HuD+ve cells

0

5

7.5

2.5

% HuD+ve cells in VZ/SVZ

GFP HuD

Scr shRNA

Lfc shRNA #1

VZ SVZ innerCM outerCM

GFP

β III tubulin

SVZ IZ

Scr shRNA

Lfc shRNA #1

Lfc shRNA #2

GFP

β III tubulin

SVZ IZ

Scr shRNA

Lfc shRNA #1

Lfc shRNA #2

Scr shRNA

Lfc shRNA #1

Lfc shRNA #2

% β III tubulin +ve in VZ/SVZ

0

2

6

2

4

8

0

NS

Exp 1

Exp 2

** *
**Figure 4.2: Genetic knockdown of Lfc inhibits neurogenesis in vivo.** Cortices were electroporated at E13/E14 with plasmids encoding both EGFP and Lfc shRNA#1, Lfc shRNA#2, or scrambled (Scr) shRNA, and analyzed four days later. (a) Photomicrographs of coronal cortical sections immunostained for EGFP (GFP, green, right panels) to show transfected cells and counterstained with Hoechst 33258 to show all nuclei (blue, left panel of each pair). VZ = ventricular zone, SVZ = subventricular zone, CM = cortical mantle. The hatched lines denote the extent of the VZ/SVZ. Scale bar = 100 μm. (b,c) Quantification of sections similar to those in (a) for migration of transfected cells, with (b) showing the percentage of cells migrating to the cortical mantle (CM), and (c) the percentage of cells in each of the cortical mantle, SVZ, and VZ. In (b), results from 3 independent experiments with Lfc shRNA#1 were combined, n = 9 and 8 brains for scrambled shRNA and Lfc shRNA#1 respectively, and results from 2 independent experiments with Lfc shRNA#2 were combined, n = 4 and 9 brains for scrambled shRNA and Lfc shRNA#2 respectively. In (c), results from 1 representative experiment are shown, n = 3 brains each for scrambled shRNA and Lfc shRNA#1. ***p<0.005, ANOVA or student’s t-test. (d) Confocal images covering the span of individual coronal sections double-labeled for EGFP (green, GFP) to detect transfected cells, and the neuronal marker HuD (red). Scale bar = 50 μm. The right panels show higher magnification micrographs of the boxed sections in the middle right panel. Scale bar = 10 um. (e,f) Quantification of the percentage of transfected HuD-positive neurons in the entire cortex (e), and in the VZ/SVZ only (f) in sections similar to those shown in (d). Results from 4 independent experiments were combined, n = 10, 5, and 9 brains for scrambled shRNA, Lfc shRNA#1 and Lfc shRNA#2, respectively. *p<0.05, **p<0.01, ANOVA. (g,h) Confocal micrographs of the VZ/SVZ of sections from cortices electroporated with Lfc shRNA#1 or a scrambled shRNA, double-labeled for EGFP (green, GFP) and βIII-tubulin (red, g) or doublecortin (red, h). The insets show the area delineated by the hatched lines at higher magnification. Arrows denote transfected double-labeled cells. Scale bar = 50μm, insets = 10 um. (i) Quantification of transfected, βIII-tubulin positive cells in the VZ/SVZ of sections similar to those shown in (g,h). In (i), n = 5 and 4 scrambled and Lfc shRNA#1 brains, respectively. *p<0.05.
Figure 4.3

(a) Immunostaining images showing Hoechst (blue) and GFP (green) in different regions of the brain. Scr shRNA and Lfc shRNA #1 treatments are indicated.

(b) Bar graph showing % migration in different regions (VZ, SVZ, CM) for Scr shRNA and Lfc shRNA #1 treatments.

(c) Immunostaining images highlighting HuD (red) and GFP (green) in the VZ/SVZ region. Scale bar indicates x5 magnification.

(d) Bar graph showing % HuD+ve cells in Exp 1 and Exp 2 for Scr shRNA and Lfc shRNA #1 treatments.

Exp 1
Exp 2

*** p < 0.001
**Figure 4.3: Lfc knockdown causes persistent changes in neurogenesis.** Cortices were electroporated at E13/E14 with plasmids encoding both EGFP and Lfc shRNA#1 or scrambled (Scr) shRNA, and analyzed at postnatal day 3. (a) Photomicrographs of coronal cortical sections immunostained for EGFP (GFP, green, right panels) to show transfected cells and counterstained with Hoechst 33258 to show all nuclei (blue, left panel of each pair). CM = cortical mantle. The hatched line denotes the extent of the VZ/SVZ. Scale bar = 100μm. (b) Quantification of sections similar to those in (a) for the percentage of transfected cells in each of the cortical mantle, SVZ, and VZ. Results from 3 independent experiments were pooled, n = 9 and 8 for scrambled shRNA and Lfc shRNA#1 respectively. ***p<0.005, ANOVA. (c) Confocal images covering the span of individual coronal sections double-labeled for EGFP (green, GFP, right panels) to detect transfected cells, and the neuronal marker HuD (red). The inset shows a higher magnification micrograph of a double-labeled cell. Scale bar = 50 μm, inset 10 μm. (d) Quantification of the percentage of total transfected HuD-positive neurons in sections similar to those shown in (c). Two independent experiments are shown, n = 7 brains each. ***p<0.005, student's t-test.
Figure 4.4: **Genetic knockdown of Lfc increases the proportion of cycling radial precursors, but does not alter astrogenesis.** (a-d) E12.5 cortical precursors were transfected with plasmids encoding EGFP plus Lfc shRNA#1, Lfc shRNA#2, or scrambled (Scr) shRNA, and cultured for varying periods of time. (a) Quantification of the percentage of transfected, Ki67-positive cells after two days in culture in two independent experiments. *p<0.05, **p<0.01, ANOVA. (b) Quantification of the percentage of transfected clones of different sizes in cultures transfected with scrambled shRNA (Scr) or Lfc shRNA#1 for 3 days in two independent experiments. (c) Immunocytochemistry for GFP (green) and GFAP (red) in a culture transfected with scrambled shRNA for 7 days. Arrows denote a transfected, double-labeled cell. Scale bar = 100 µm. (d) Quantification of the percentage of transfected, GFAP-positive cells in cultures similar to that shown in (c). Two independent experiments were pooled, p>0.05, student's t-test. (e-m) Cortices were electroporated at E13/E14 with plasmids encoding both EGFP and Lfc shRNA#1 or scrambled (Scr) shRNA, and analyzed at varying timepoints. (e) Confocal micrographs of the VZ/SVZ of a cortical section from an embryo that was electroporated with Lfc shRNA#1, labeled with BrdU one day later, and then double-labeled for EGFP (green, GFP), Ki67 (blue), and BrdU (red) 24 hours following BrdU injection. Arrows denote transfected triple-labeled cells. Scale bar = 50 µm. (f) Quantification of transfected, Ki67-positive cells in the VZ/SVZ of sections similar to that shown in (e). Results are pooled from 3 independent experiments, n = 10 and 11 brains for scrambled (Scr) shRNA and Lfc shRNA#1. ***p<0.005. (g) Quantification of the percentage of transfected BrdU-positive cells that were also Ki67-positive in the VZ/SVZ of sections similar to that shown in (e). Results of 2 independent experiments are shown, n = 7 brains each for scrambled (Scr) shRNA and Lfc shRNA#1. ***p<0.005. (h) Confocal micrographs of the VZ/SVZ of cortical sections that were immunostained for GFP (green) and Pax6 (red) two days following electroporation. Arrows denote double-labeled cells. Scale bar = 50 µm. (i) Quantification of the percentage of transfected, Pax6-positive cells in the VZ/SVZ of sections similar to those in (h). Results are pooled from 3 independent experiments, n = 8 and 7 brains for scrambled shRNA and Lfc shRNA#1, respectively. ***p<0.005. (j) Photomicrograph of a cortical section electroporated with Lfc shRNA#1 and immunostained for GFP (green) and BLBP (green) 4 days post-electroporation. The arrow indicates the long BLBP-positive process of a transfected radial precursor. Scale bar = 50 µm.
μm. (k) Quantification of the percentage of transfected Pax6-positive cells in the SVZ 2 days following electroporation. Results are pooled from 2 independent experiments, n = 7 and 6 brains for scrambled shRNA and Lfc shRNA#1. *p<0.05. (l) Confocal micrographs of the VZ/SVZ of cortical sections that were immunostained for GFP (green) and the basal progenitor marker Tbr2 (red) 2 days following electroporation. Arrows denote transfected, double-labeled cells. Scale bar = 50 μm. (m) Quantification of the percentage of Tbr2-positive cells in the VZ/SVZ of sections similar to those in (l). Results of 2 independent experiments are shown, n = 6 and 5 brains for scrambled shRNA and Lfc shRNA#1, respectively. *p<0.05.
Figure 4.5

(a) Western blot analysis showing Tctex-1, ERK1/2, and βIII tubulin protein expression at different stages (E12 to E18). 
(b) Graph comparing % βIII tubulin+ve cells for Scr shRNA and Tctex-1 shRNA#1 across experiments 1-3. 
(c) Immunofluorescence images depicting HuD+ve cells in VZ/SVZ regions with Hoechst and GFP staining. 
(d) Graph showing % migration to CM for Scr shRNA and Tctex-1 shRNA#1. 
(e) Graph comparing % HuD+ve cells for Scr shRNA and Tctex-1 shRNA#1. 
(f) Graph showing % HuD+ve cells in VZ/SVZ. 
(g) Immunofluorescence images for Scr shRNA and Tctex-1 shRNA#1 in CM and VZ/SVZ regions. 
(h) Graph comparing % HuD+ve cells in VZ/SVZ for Scr shRNA and Tctex-1 shRNA#1. 
(i) Graph showing % migration to CM for Scr shRNA and Tctex-1 shRNA#1. 
(j) Graph comparing % migration to CM for different stages (II/III, IV/V, VI).
Figure 4.5: Genetic knockdown of Tctex-1, a negative regulator of Lfc, promotes cortical neurogenesis. (a) Western blot for Tctex-1 in cortical lysates from embryonic day 11 (E11) to E18. The blot was reprobed for Erk1/2 as a loading control. (b) Quantification of transfected, βIII-tubulin-positive neurons in precursor cultures cotransfected with plasmids encoding EGFP and Tctex-1 shRNA#1 or scrambled shRNA (Scr) for 3 days. Three independent experiments are shown. *p<0.05, student's t-test. (c-j) Cortices were electroporated at E13/E14 with plasmids encoding both EGFP and Tctex-1 shRNA#1, Tctex-1 shRNA#2, or scrambled (Scr) shRNA, and analyzed (c-f) four days later or (g-j) at postnatal day 3. (c) Confocal images covering the span of individual coronal sections double-labeled for EGFP (green, GFP) to detect transfected cells, and the neuronal marker HuD (red). The insets show double-labeled cells (arrows) at higher magnification. Scale bar = 50 μm, insets = 10 μm. (d) Quantification of sections similar to those in (c) for migration of transfected cells to the cortical mantle. Results from 4 independent experiments were combined, n = 10, 7, and 5 brains for scrambled shRNA, Tctex-1 shRNA#1, and Tctex-1 shRNA#2, respectively. **p<0.005, ANOVA. (e,f) Quantification of the percentage of transfected HuD-positive neurons in the entire cortex (e), and in the VZ/SVZ (f) in sections similar to those shown in (c). Results from 2 independent experiments were combined, n = 5, 7, and 3 brains for scrambled shRNA, Tctex-1 shRNA#1 and Tctex-1 shRNA#2, respectively. **p<0.01, ANOVA. (g) Photomicrographs of coronal cortical sections from brains electroporated at E13/E14 and immunostained at P3 for EGFP (GFP, green, right panels) to show transfected cells and counterstained with Hoechst 33258 to show all nuclei (blue, left panel of each pair). The hatched line denotes the extent of the VZ/SVZ. Scale bar = 50μm. (h,i) Quantification of the percentage of transfected HuD-positive neurons in the entire cortex (h), and in the VZ/SVZ (i) in sections similar to those shown in (g). n = 7 and 4 for scrambled shRNA and Tctex-1 shRNA #1, respectively. **p<0.01, ***p<0.005, student's t-test. (j) Quantification of transfected cells that migrated to neuronal layers II/III, IV-V and VI in cortices electroporated at E13.5 with scrambled (Scr) shRNA or Tctex-1 shRNA#1 and analyzed at postnatal day three. n = 7 and 4 for scrambled and Tctex-1 shRNA#1, respectively. *p<0.05, ***p<0.001, student’s t-test.
Figure 4.6

(a) Fluorescence images of GFP and Ki67 expression in cells treated with Scr shRNA or Tctex-1 shRNA#1. Scale bars: 100 μm.

(b) Bar graph showing the percentage of Ki67+ve cells in Exp 1 and Exp 2 for Scr shRNA and Tctex-1 shRNA#1. Bars with ** indicate significance at p < 0.01 and *** at p < 0.001.

(c) Fluorescence images of GFP and Pax6 expression in cells treated with Scr shRNA or Tctex-1 shRNA#1. Scale bars: 100 μm.

(d) Bar graph showing the percentage of Pax6+ve cells in Exp 1 and Exp 2 for Scr shRNA and Tctex-1 shRNA#1. Bars with ** indicate significance at p < 0.01.

(e) Fluorescence images of GFP, Pax6, and BLBP expression in cells treated with Scr shRNA or Tctex-1 shRNA#1. Scale bars: 100 μm.
Figure 4.6: **Genetic knockdown of Tctex-1 decreases the proportion of cycling radial precursors.** Cortices were electroporated at E13/E14 with plasmids encoding both EGFP and Tctex-1 shRNA#1 or scrambled (Scr) shRNA, and analyzed 2 days later. (a) Confocal micrographs of the VZ/SVZ of electroporated cortical sections that were double-labeled for EGFP (green, GFP), and Ki67 (red). Arrows denote transfected double-labeled cells. Scale bar = 50 μm. (b) Quantification of transfected, Ki67-positive cells in the VZ/SVZ of sections similar to those shown in (a). Results are shown from 2 independent experiments, n = 5 and 6 brains for scrambled (Scr) shRNA and Tctex-1 shRNA#1. **p<0.01, ***p<0.005, student's t-test. (c) Confocal micrographs of the VZ/SVZ of cortical sections that were immunostained for GFP (green) and Pax6 (red). Arrows denote double-labeled cells. Scale bar = 50 μm. (d) Quantification of the percentage of transfected, Pax6-positive cells in the VZ/SVZ of sections similar to those in (c). Two independent experiments are shown, n = 5 and 6 brains for scrambled shRNA and Tctex-1 shRNA#1, respectively. **p<0.01, student's t-test. (e) Photomicrograph of sections from cortices electroporated with scrambled shRNA or Tctex-1 shRNA#1 immunostained for GFP (green) and BLBP (red) 2 days post-electroporation. The bottom two panels show higher magnification images of the sections denoted with hatched lines in the top two panels. Arrows indicate long BLBP-positive processes of transfected radial precursors. Scale bar = top two panels, 50 μm, bottom two panels, 10 μm.
Figure 4.7

(a) Immunohistochemistry for HuD (dsRed) and GFP showing effects of Tctex-1 shRNA and Lfc shRNA on HuD+ve cells in VZ/SVZ.

(b) Bar graph showing % migration to CM for different groups: Scr shRNA + Scr shRNA, Tctex-1 shRNA + Scr shRNA, Tctex-1 shRNA + Lfc shRNA.

(c) Bar graph showing % HuD+ve cells in VZ/SVZ for different groups: Scr shRNA + Scr shRNA, Tctex-1 shRNA + Scr shRNA, Tctex-1 shRNA + Lfc shRNA.

(d) Bar graph showing % HuD+ve cells in VZ/SVZ for different groups: Scr shRNA + Scr shRNA, Tctex-1 shRNA + Scr shRNA, Tctex-1 shRNA + Lfc shRNA.
Figure 4.7: Coincident silencing of Lfc rescues the Tctex-1 knockdown phenotype. Cortices were coelectroporated at E13/E14 with plasmids encoding Tctex-1 shRNA #1 plus EGFP and either Lfc shRNA#1 plus dsRed or scrambled shRNA plus dsRed, and analyzed 4 days later. As an additional control, some cortices were electroporated with scrambled shRNA plus EGFP and scrambled shRNA plus dsRed. (a) Confocal micrographs of a cortical section double-labeled for GFP (green) and HuD (blue), and the dsRed fluorescence visualized without amplification. The boxed section in the merged picture is blown up in the bottom panel. The arrows indicate a triple-labeled cell. (b) Quantification of sections similar to that in (a) for migration of co-transfected cells to the cortical mantle. Results from 2 independent experiments were combined, n = 4,5, and 5 brains for scrambled shRNA + scrambled shRNA, Tctex-1 shRNA#1 + scrambled shRNA, and Tctex-1 shRNA#1 + Lfc shRNA#1, respectively. **p<0.01, ANOVA. (c,d) Quantification of the percentage of transfected HuD-positive neurons in the entire cortex (c), and in the VZ/SVZ (d) in sections similar to that shown in (a). Results from 3 independent experiments were combined, n = 3, 7, and 6 brains for scrambled shRNA + scrambled shRNA, Tctex-1 shRNA#1 + scrambled shRNA, and Tctex-1 shRNA#1 + Lfc shRNA#1, respectively. *p<0.05, **p<0.01, ANOVA.
Figure 4.8

(a) Lfc, α-tubulin, Hoechst, and merge images showing metaphase and anaphase/telophase.

(b) Bar graph showing the percentage of mitotic spindles oriented 0-15° from the VZ for Scr shRNA and Lfc shRNA #1.

(c) Bar graph showing the percentage of mitotic spindles oriented 0-15° from the VZ for E14 + 2dpe.
Figure 4.8: *Lfc regulates mitotic spindle orientation in dividing cortical precursors.*  (a) Photomicrographs of cultured cortical precursors immunostained for Lfc (red) and α-tubulin (green) to detect microtubules, and counterstained with Hoechst 33258 (blue) to visualize dividing chromosomes. Scale bar = 10 μm.  (b) Confocal micrographs of the ventricular surface of a cortical section electroporated 2 days earlier with scrambled or Lfc shRNA#1, and then immunostained for GFP (green) to detect transfected cells, γ-tubulin (red) to visualize centrosomes, and phospho-histone 3 (pH3, not shown) to detect dividing chromosomes, and then counterstained with Hoechst 33258 (blue) to detect DNA. The dotted line denotes the spindle orientation of transfected cells in metaphase or anaphase/telophase. Scale bar = 10 μm.  (c) Quantification of the percentage of transfected dividing apical precursors in metaphase or anaphase/telophase with a vertical mitotic spindle orientation determined from micrographs similar to that seen in (b). Results from 4 independent experiments were pooled where a total of 89 scrambled shRNA and 130 Lfc shRNA#1-transfected cells were counted, and where n=10 and n=11 brains each *p<0.05, students t-test.
Chapter 5: Discussion

5.1 SHP-2 regulates the timing and extent of neurogenesis and gliogenesis during cortical development

Little is known about how environmental cues such as local growth factors instruct multipotent precursor cells to generate one cell type at the expense of another. Here, we provide evidence that growth factor-mediated activation of SHP-2 provides such a decisional switch, instructing embryonic cortical precursors to generate neurons rather than glial cells, even in the presence of low levels of gliogenic cytokines. This mechanism is essential for the appropriate timed genesis of neurons versus astrocytes, and for determining the relative numbers of these two cell types. Such a growth factor-regulated intracellular switch provides a mechanism for integrating intrinsic cellular programs with the external neural environment, thereby ensuring appropriate neural development. Moreover, perturbation of this regulatory mechanism in genetic disorders such as Noonan Syndrome is likely to perturb the ratio of neurons versus glia and thus contribute to cognitive dysfunction.

During development of the mammalian nervous system, cell genesis is a timed event, with neurons generated first, and glial cells second. Within the murine cortex, these neurogenic and gliogenic periods are temporally distinct; neurogenesis occurs from approximately E12-E17 while astrocyte formation is largely postnatal. Many of the previous studies examining the nature of the underlying timer have focused upon positive, inductive mechanisms (Bonni et al., 1997; Burrows et al., 1997; Sun et al., 2005; Takizawa et al., 2001; He et al., 2005). For example, the gliogenic cytokine cardiotrophin-1 is synthesized and secreted by newly-born neurons, and thereby acts to promote the timed genesis of astrocytes (Barnabé-Heider et al., 2005), and the tyrosine kinase receptor EGFR, which also promotes gliogenic competence, is induced on cortical precursors and asymmetrically-segregated to their glial progeny (Burrows et al., 1997; Sun et al., 2005). Here, we show that appropriate timed gliogenesis also depends upon a SHP-2-mediated suppressive signal that inhibits competent precursors from prematurely generating astrocytes. The presence of such suppressive signals may be a biological necessity, since we have previously shown that at least some cortical precursors are competent to generate astrocytes during the neurogenic period (Barnabé-Heider et al., 2005), and others have shown that the gliogenic cytokines
neuropoietin and cardiotrophin-like cytokine are expressed in embryonic mouse neuroepithelia (Uemura et al., 2002; Derouet et al., 2004).

5.1.1 Summary of Major conclusions

The data presented in Chapter 3 support three major conclusions. First, the experiments using SHP-2 shRNA indicate that endogenous SHP-2 is essential for the normal genesis of cortical neurons and astrocytes both in culture and within the environment of the embryonic cortex. Genetic knockdown of SHP-2 leads to a delay in neurogenesis and ultimately a perturbation in neuronal location. It also causes inappropriate precocious formation of astrocytes during the embryonic neurogenic period, and leads to a robust increase in the number of astrocytes generated. Second, as shown in the clonal experiments, SHP-2 mediates these effects by instructing neural precursors to generate neurons rather than astrocytes, thereby ensuring that precursors that are biased to a neuronal fate do not attempt to become glia even in the presence of cytokines. This occurs via two dissociable mechanisms; SHP-2 activates the neurogenic Ras-MEK-C/EBP pathway, and at the same time inhibits the gliogenic gp130-JAK-STAT pathway. Third, the constitutive activation of SHP-2 that occurs in Noonan Syndrome causes a large reduction in astrocyte formation, and a coincident increase in neuronal number in the forebrain and hippocampus. These effects are seen both following ectopic expression of a Noonan Syndrome SHP-2 allele and, importantly, in a Noonan Syndrome mouse model carrying the same activated allele. Together these data indicate that SHP-2 regulates the timing and extent of neurogenesis versus gliogenesis by acting as a growth factor-regulated switch to bias cortical precursor cells to one fate and against another. Moreover, perturbations in the ratio of neurons versus glia following constitutive SHP-2 activation may contribute to the cognitive dysfunction observed in Noonan Syndrome individuals.

5.1.2 How SHP-2 regulates neurogenesis and gliogenesis

From a mechanistic perspective, how does SHP-2 function to regulate the extent and timing of neurogenesis versus gliogenesis? Our data indicate that SHP-2 instructs cortical precursors to generate neurons rather than astrocytes by activating the MEK-ERK pathway and by negatively-regulating the gp130-JAK-STAT pathway, respectively. How SHP-2
mediates Ras activation, and MEK-ERK, in this context remains unclear. Current literature suggests that SHP-2 can activate Ras by indirectly activating or preventing inactivation of Src-family kinases through various mechanisms (Zhang et al, 2004; reviewed in Neel et al, 2003; Roskoski, 2005) or by dephosphorylating and inactivating Sprouty, an inhibitor of the Ras-MEK-ERK signaling pathway (Hanafusa et al, 2004; Hanafusa et al, 2004, reviewed in Neel et al, 2003; Dance et al., 2008). Furthermore, SHP-2 activation and tyrosyl phosphorylation leads to association with other adapter molecules such as FRS-2, Grb-2-Sos, and Gab1 and Gab2 (Hadari et al, 1998; Schlessinger, 2000; Cunnick et al, 2001; Neel et al, 2003; Toshiyuki et al, 2003; Montagner et al, 2005), and availability of these molecules in cortical precursors could also play a role in MEK-ERK-mediated neurogenesis. Finally, although we have shown that SHP-2 is required for cortical neurogenesis, Shc adapter proteins which also regulate RTK-mediated MEK-ERK activation, could also work in conjunction with SHP-2 in regulation of neuronal differentiation. (Conti et al, 2001) In this regard, we have previously shown that activation of the MEK-ERK-C/EBP pathway is essential for normal cortical neurogenesis both in culture and in vivo (Ménard et al., 2002; Barnabe-Heider et al., 2003; Paquin et al., 2005). Interestingly, our work indicated that MEK is essential for neurogenesis but has no role in gliogenesis, while the C/EBPs, like SHP-2, both promote neurogenesis and inhibit gliogenesis. The C/EBPs promote neurogenesis by directly promoting transcription of neuron-specific genes such as Tα1 α-tubulin (Menard et al., 2002), but it is not yet clear how they inhibit gliogenesis.

How does SHP-2 suppress gliogenesis? Here we suggest that SHP-2 inhibits gliogenesis by directly dephosphorylating and inactivating components of the gp130 signaling pathway. Previous studies have implicated SHP-2 as a negative regulator of gp130-JAK-STAT signaling by showing that tyrosine 759 of gp130, the binding site shared by SHP-2 and another inhibitor of JAK, SOCS3, is involved in inhibition of IL-6 signaling. (Ohtani et al, 2000; Bartoe and Nathanson, 2002; Lehmann et al, 2003; Ernst and Jenkins, 2004; Fischer et al, 2004; Clahsen et al, 2005). SOCS-3 belongs to a family of suppressors of cytokine signaling (SOCS) proteins that are induced transcriptionally by STAT signaling, and act as classic feedback inhibitors of IL-6-related signaling (Endo et al, 1997; Starr et al, 1997; Naka et al, 1997). Both SHP-2 and SOCS3 have been shown to independently contribute to the negative regulation of gp130-mediated JAK-STAT signaling (Bartoe and Nathanson, 2002),
but only SHP-2 interacts with LIFRβ at tyrosine 974 (Clahsen et al., 2005). We speculate that SHP-2 has an earlier role in regulating this pathway when gliogenic cytokines are expressed at low levels, whereas STAT3-induced SOCS may have a desensitizing role when gliogenic cytokines are expressed at higher levels. Recently, overexpression of SOCS3 has been shown to inhibit astrocyte formation and to promote the maintenance of neural stem cells (Cao et al., 2006).

The studies mentioned above lead to speculation that SHP-2 may negatively regulate JAK-STAT signaling via interactions with gp130, JAK (Ernst and Jenkins, 2004) or by dephosphorylation of STATs (Chen et al., 2003). Our preliminary and unpublished data demonstrate that SHP-2 is found in a complex with many components of the gp130-JAK-STAT pathway in precursors treated with CNTF (data not shown), suggesting that it could directly dephosphorylate and inhibit gliogenic proteins. Preliminary in vitro phosphatase assays demonstrate that SHP-2 can at least directly dephosphorylate phosphotyrosine residues on the gp130 co-receptor (data not shown), one of the important players in cortical gliogenesis (Bonni et al., 1997), in agreement with the observation that the phosphatase activity of SHP-2 is required for its regulation of astrocyte formation. Consistently, overexpression of SHP-2 in CNTF-responsive cell lines such as SHSY5Y results in decreased phosphoSTAT3 signal when compared to control transfected cells (data not shown). Could its role in regulation of Ras signaling also contribute to the negative regulation of gliogenic signaling? In other systems, Ras signaling has been shown to downregulate STAT3 activity at the posttranslational level (Yeh et al., 2008), while the downstream C/EBP transcription factors may repress gliogenic genes or compete with gliogenic transcription factors for transcriptional co-activators. Thus, RTK signaling via the SHP-2-Ras-MEK-ERK-C/EBP pathway serves to promote neurogenesis and at the same time to inhibit gliogenesis via multiple potential mechanisms.

While our data indicate that one important function of SHP-2 is to regulate the timing and extent of neural cell genesis, a second perhaps equally important function is to act as a switch to ensure that differentiating cells adopt only a single cell fate. The significance of such a role is indicated by our finding that in the clonal experiments described here, a small number of cells transfected with SHP-2 shRNA displayed a mixed neuronal/glial phenotype, something never seen in transfected, control cells. In this regard, while precedent exists for
transcription factors such as the C/EBPs (Ménard et al., 2002; Paquin et al., 2005) and neurogenic bHLHs (Nieto et al., 2001; Sun et al., 2001) functioning to promote neurogenesis at the expense of gliogenesis, SHP-2 is the first signaling protein identified to perform such a function. We propose that these findings provide a molecular explanation for a previous study where PDGFR signaling, which is neurogenic for cultured cortical precursors, was dominant over the gliogenic cytokine CNTF (Park et al., 1999). It will be interesting to determine whether SHP-2 also functions as a cell fate switch in other stem cell populations, such as hematopoietic and embryonic stem cells, where it regulates self-renewal and differentiation (Qu et al., 2001; Chan et al., 2003; Zou et al., 2006). Interestingly, SHP-2 also functions as a molecular switch in ES cells cultured under neurogenic condition, governing self-renewal and differentiation via the bidirectional regulation of ERK and STAT3 activations. In these studies, SHP-2 deletion leads to decreased neurogenesis and more efficient self-renewal (reviewed in Feng, 2007).

What are the growth factors that signal through receptor tyrosine kinases to activate SHP-2 and promote neurogenesis? Previous work has identified a number of candidate receptor tyrosine kinase ligands, including FGF2, neurotrophins, PDGF and IGF1. Specifically, FGF2 is mitogenic for cortical precursors (Lukaszewicz et al., 2002) and is essential for normal neurogenesis (Raballo et al., 2000). Cortical precursors express the neurotrophin receptors TrkB and TrkC, and ablation of either TrkB or the neurotrophins BDNF and/or NT-3 perturb the development of these precursors (Barnabé-Heider and Miller, 2003; Bartkowska et al., 2007). Cultured cortical precursors express PDGFRs and enhance neurogenesis in response to PDGF (Johe et al., 1996; Williams et al., 1997; Park et al., 1999). Finally, overexpression of IGF1 from the nestin promoter causes increased proliferation and neuron numbers in the embryonic cortex (Popken et al., 2004). What are the sources of these growth factors during neurogenesis? Cortical precursors of the VZ/SVZ themselves express BDNF, NT-3 (Maisonpierre et al., 1990; Fukumitsu et al., 1998), and FGF2 (Raballo et al., 2000). Intriguingly, endothelial cells also express BDNF (Kim et al., 2004), FGF2 (Albuquerque et al., 1998), and PDGF (Arkonac et al., 1998), and embryonic endothelial cells have recently been shown to promote self-renewal and neurogenesis in neural stem cells (Shen et al., 2004). Thus, it is likely that cortical precursors are exposed to diverse ligands from multiple sources, and signaling via these growth factors could regulate SHP-2 to
promote neurogenesis and inhibit gliogenesis. More recently, integrin signaling has also been implicated in the regulation of ES-cell derived neural stem cells, more specifically in regulation of neurogenesis and neuronal migration (Andressen et al., 2005). But why does SHP-2-mediated ERK activation promote differentiation and not proliferation in response to these multifunctional growth factors in cortical precursors? Although timing and cellular context may determine this outcome, a recent study performed in PC12 cells suggests that sustained ERK activation leads to differentiation, and is regulated by SHP-2, while acute ERK activation leads to proliferation. In this scenario, the neurotrophin NGF leads to neuronal differentiation while EGF stimulation leads to increased proliferation (D’Alessio et al., 2007). How these two factors differentially activate SHP-2 and the Ras-MEK-ERK to promote these effects are unclear, but may be cell-context dependent, and may largely depend on receptor membrane environment, for example clustering of some receptors in lipid rafts, or presence of receptors at cell bodies versus radial processes.

We therefore propose a model where, during the embryonic period, cortical precursors are biased to generate neurons by expression of bHLHs such as neurogenins (Sun et al., 2001; Nieto et al., 2001), but that the precise timing and number of neurons is then regulated by ligands that activate receptor tyrosine kinases. These activated receptors promote neurogenesis by inducing the neurogenic MEK-ERK-C/EBP pathway, and data presented here indicate that SHP-2, which is expressed at high levels in these neurogenic precursors, is an integral upstream component of this pathway. During the same developmental window, these precursors are exposed to limiting levels of cytokines such as neuropoietin 1 and cardiotrophin-like cytokine (Uemura et al., 2002; Derouet et al., 2004), which can activate the gliogenic gp130-JAK-STAT pathway. Our data indicate that SHP-2 suppresses this gliogenic pathway, thereby ensuring that precursors generate only neurons and not glia during the neurogenic period. As embryonic development proceeds, these newly-born neurons synthesize and secrete cardiotrophin-1, and cytokine levels within the vicinity of cortical precursors increase substantively. Over this same time period, cortical precursors substantially decrease their expression of SHP-2, and become sensitized to cytokines via a variety of intrinsic mechanisms, including increased expression of EGFR (Burrows et al., 1997; Sun et al., 2005) and/or components of the gliogenic JAK-STAT pathway (He et al., 2005), and demethylation of astrocyte-specific genes (Takizawa et al.,...
2001; Fan et al., 2005). They also upregulate the astrogenic NFIs transcription factors (Deneen et al., 2006). Under these new conditions, SHP-2 cannot completely suppress gliogenesis, but instead functions to regulate the number of astrocytes that are generated by negatively regulating gp130-JAK-STAT signaling. Moreover, robust gp130-JAK-STAT activation in response to elevated cardiotrophin-1 serves to suppress neurogenesis via an as-yet-undefined mechanism (Barnabé-Heider et al., 2005). Thus, SHP-2 functions as an essential component of a growth factor regulated signaling mechanism that determines the timing and extent of neurogenesis versus gliogenesis in the developing cortex.

5.1.5 Studies published since April 2007 support our findings

Interestingly, a few studies using alternative approaches were published shortly after our publication, and corroborate our results. For example, overexpression of SHP-2 in a hippocampal neural stem cell line led to increased neuronal differentiation and maturation, while dominant negative forms of SHP-2 had the opposite effect (Kim et al., 2007). While SHP-2ΔP increased astrocyte formation in our study, there was no effect on neurogenesis. The differences may be due to low expression of the transgene at early timepoints of analysis in our system, or to differences between cortical and hippocampal neural stem cells.

Another group’s parallel investigation into the role of SHP-2 in cortical development yielded similar conclusions to the ones presented in this study (reviewed in Coskun et al., 2007). Using a neural precursor-specific conditional SHP-2 knockout, the authors showed that SHP-2 is required for neurogenesis and for the suppression of gliogenesis until postnatal stages, and that it does this by promoting and suppressing ERK and STAT3 activation respectively (Ke et al., 2007), just as we proposed. The authors additionally documented a role for SHP-2 in FGF2-mediated stimulation of self-renewing proliferation, in part by controlling Bmi1 expression (Ke et al., 2007). In our study, we did not report effects on proliferation when SHP-2 expression was reduced, although clone sizes were consistently, but not significantly, smaller in these conditions when compared to transfected controls. Limitations in our gene silencing approach could explain this, where more time may be required for complete SHP-2 knockdown, and where residual SHP-2 activity most likely remained. We may also have missed this role due to the relatively late timing of our manipulation in vivo. Our in utero electroporations are performed at E13, whereas the other
group was able to knockout SHP-2 as early as E10 using a nestin-driven cre transgene. Furthermore, although the authors also documented a decrease in neurogenesis and increase in astrocyte formation, this could be due to non-cell autonomous defects, subsequent to self-renewal and survival, whereas our acute knockdown experiments suggest that the effect of SHP-2 on neurogenesis and gliogenesis is cell autonomous.

5.2 Neuro-cardio-facial-cutaneous (NCFC) syndromes

5.2.1 Neural perturbations in Noonan Syndrome

An equally important major conclusion of our study is that the constitutive activation of SHP-2 seen in Noonan Syndrome leads to perturbations in cortical cell fate decisions, causing enhanced neurogenesis and a robust decrease in gliogenesis. Interestingly, when we compared adult D61G/+ mice brains with wildtype counterparts, we found that they are smaller (unpublished), consistent with important changes in cell genesis or dendritic arborization. We observed these perturbations in a mouse model of Noonan Syndrome that phenocopies the human condition with regard to all parameters so far examined (Araki et al., 2004). Moreover, our in utero electroporation studies argue that these effects are largely cell-autonomous, with the D61G SHP-2 allele directly perturbing precursor cell fate decisions, and are not an indirect consequence of other changes in the mouse model. Studies of this Noonan Syndrome mouse model argue that the cell genesis perturbations may cause cognitive defects. These animals display deficits in hippocampal spatial learning, as monitored by the Morris water maze (unpublished, communication with B.G. Neel), and we have documented perturbed hippocampal cell genesis. On the basis of these findings, we suggest that perturbations in cell genesis during embryonic and early neonatal development lead to alterations in cell survival, circuit formation, and subsequent neural function. Ultimately, whether perturbations in cell genesis can result in cognitive impairments would of course have to be tested using conditional and inducible forms of the Noonan alleles. For example, what is the behavioral outcome if a Noonan-associated allele is only active in precursors during embryogenesis, but turned off upon differentiation?

Similar perturbations remain to be confirmed in the human syndrome. While it is clear that many of these individuals display learning disabilities and mental retardation (Noonan et al., 1994; Yoshida et al., 2004; Jongmans et al., 2005; Lee et al., 2005), there
have only been a few brain imaging studies reported. However, those few studies do indeed indicate that there are perturbations, including cortical dysplasia, cortical atrophy, and cerebellar malformations (Saito et al., 1997; Görke, 1980; Ball and Peiris, 1982; Heye and Dunne, 1995). Since the mouse model that we studied here has been shown to phenocopy the human condition with regard to more well-studied aspects of the syndrome such as craniofacial abnormalities, myeloproliferation, and cardiac defects, then we believe that neural defects similar to those we documented are likely to occur in the human condition.

5.2.2 Neural perturbations in other NCFC disorders

Neurofibromatosis (NF), Costello and cardio-facial-cutaneous (CFC) syndromes, are classified in the same family of genetic disorders as Noonan syndrome, have recently been shown to be caused by activating mutations in the NF-1-Ras-Raf-MEK-ERK pathway (reviewed in Bentires-Alj et al., 2006; Denayer et al., 2007), and present with severe mental retardation and variable seizures. The recent study of a CFC patient cohort summarizes important neurological examination and imaging findings, which include macrocephaly, hydrocephaly, and other structural abnormalities (Yoon et al., 2007). Similarly, Costello patients present with neurological abnormalities including ventricular dilation, brain atrophy, and other structural anomalies (reviewed in Delrue et al., 2003). This is consistent with the possibility that neural cell genesis is perturbed in these patients. Thus, we propose that the SHP-2-MEK-ERK-Rsk-C/EBP pathway we have defined as important for neural cell fate determination is essential for normal human brain development.

A G12V H-Ras knock-in Costello mouse model has recently been generated, and displays mild neurological and behavioral deficits, including spatial learning deficits (Viosca et al., 2008). Although the cellular etiology of these deficits was not investigated extensively, no gross neural abnormalities were reported. Interestingly, the overexpression of H-Ras or of a Costello-associated activated H-RasG12V/S mutants in neural precursors leads to hyperproliferation of precursors and precocious astrocyte formation in culture and in vivo (Paquin et al., submitted), outcomes that differ from the effects observed when SHP-2 activity is increased. Although these were overexpression experiments, a conditional neurofibromatosis (NF) mouse model where the NF-1 tumor suppressor and negative regulator of Ras signaling is inactivated in neural precursors results in similar perturbations,
with increased precursor proliferation and self-renewal, as well as increased astrocyte formation and abnormal neuronal differentiation, mimicking the effects of constitutively active KRasG12D (Hegedus et al., 2007). Not surprisingly, inactivating nf1 mutations and activating mutations or increased copy numbers of the k-ras and Raf-1 (c-raf) genes have all been implicated in glioma formation and maintenance (Lyustikman et al., 2008; Jeuken et al., 2007; Uhrbom et al., 2002; Gutmann et al., 2008), in agreement with the findings that glial precursors are upregulated in some of these mouse models.

So, why do the activated SHP-2 results differ? First, it is possible that other mutants are more potent than the SHP-2 mutant used in this study, and lead to increased ERK activation, as well as other pathways including PI-3K which, in cooperation with MEK-ERK signaling, regulate survival and proliferation in many cell types. It would be interesting to determine whether the various NCFC-associated mutants behave differently biochemically with respect to activation of those pathways. It is also likely that, given its position in the RTK signaling pathway, SHP-2 differs from Ras and downstream effectors in its susceptibility to ERK-dependent feedback inhibition, suggesting that activation of MAPK signaling proteins can be dampened differentially by feedback regulators of this pathway.

SHP-2 is known to promote the sustained activation of RAS-MEK-ERK as well as PI-3K/Akt signaling, which have been implicated in neural precursor proliferation and survival. Consistently, we also document a small increase in the survival of cortical precursors transfected with a wildtype or activated form of SHP-2 in culture, an increase that cannot account for the increased neurogenesis. However, we did not observe increased precursor proliferation or significant changes in clone size from these transfected precursors. On the contrary, there was a trend towards smaller clone sizes, which is consistent with the increase in neuronal differentiation. Furthermore, a small increase in cell density was observed in two brain regions of the Noonan syndrome mouse model at birth, which could be attributed to increased cell survival or to early effects on precursor proliferation.

Its role as a negative regulator of gp130/JAK/STAT signaling probably confers on SHP-2 its unique ability among Ras pathway signaling proteins to suppress gliogenesis, by down-regulating STAT3 activation, in agreement with data presented in our studies and those of Ke and colleagues (Ke et al., 2007). As discussed in chapter 1, STAT3 signaling may regulate both self-renewal and gliogenesis (Yoshimatsu et al., 2006). As such, increasing
SHP-2 activity could result in reduced proliferation and astrocyte formation. Perhaps increasing MEK-ERK activation is not sufficient to promote proliferation in the absence of STAT3 signaling or other signals activated by potent Ras activation, and this may be context-dependent, as remains to be investigated.

But how can increased Ras expression and activity lead to precocious astrocyte formation, as assessed by increased early expression of the late astrocytic marker GFAP? As discussed in chapter 1, FGFR signaling (and perhaps EGFR signaling as well) contributes to the epigenetic regulation at the gfap promoter. In this case, increased Ras activation may be potentiating these effects, leading to the precocious expression of gfap in cycling precursors. It has not been determined whether the transformed cells are true astrocytes or transformed precursors, for example by determining whether they co-express markers of neural stem cells such as nestin or other astrocytic markers. Interestingly, these results are reminiscent of a study where increasing EGFR levels in precursors led to increased proliferation and premature astrocyte formation (Burrows et al., 1997). An alternative explanation may be that activated Ras mimicks the induction of an FGF-mediated subpopulation of gliogenic precursors, changing their cell cycle dynamics and causing the premature differentiation of astrocytes. It would be interesting to see if Ras-transfected precursors have enhanced expression of EGFRs or altered DNA methylation.

Whatever the explanation, it is clear that perturbations in RTK-Ras-MEK-ERK signaling lead to perturbed cell genesis during cortical development, and that resulting imbalances in neuronal and glial numbers, whichever way shifted, could lead to similar cognitive outcomes. For example, LEOPARD syndrome is associated with phosphatase-dead SHP-2 mutations, but closely overlaps with Noonan syndrome which is caused by activating mutations. Interestingly, we found that a phosphatase deletion, dominant-negative form of SHP-2 increased astrocyte formation but had no effect on neurogenesis. This suggests that the role of SHP-2 as an adaptor protein may be sufficient to maintain normal neurogenesis in an overexpression context. It remains to be seen whether LEOPARD syndrome-associated SHP-2 mutations act similarly. Mutations in the exchange factor SOS, KRas and Raf-1 have also been recently implicated in Noonan Syndrome, again, suggesting that different types of perturbations in cell genesis could lead to similar phenotypes.
5.2.3 Cell genesis perturbations during cortical development can lead to mental retardation

Little is known about how genetic perturbations lead to mental retardation. Here, we have identified a signaling protein, SHP-2, that plays a key role in allowing environmental cues such as growth factors to instruct multipotent precursor cells to generate one cell type versus another during development. Moreover, we have shown that constitutive activation of this protein in Noonan Syndrome causes aberrant neural development, providing a potential explanation for the cognitive dysfunction observed in this disorder. These findings raise the possibility that genetically-defined perturbations in cell genesis may well be an underlying deficit in a number of genetic developmental disorders that affect cognitive function as has been proposed, but not conclusively shown, in disorders such as autism (DiCicco-Bloom et al, 2006).

In this regard, Noonan syndrome has been classified under the broad umbrella of autistic spectrum disorders (ASD) along with other disorders sharing core symptom domains including deficits in communication, abnormal social interactions, and restrictive and/or repetitive interests and behaviors. (Ghaziuddin et al, 1994; Artigas-Pallared et al, 2005). Other examples include Rett syndrome, where the repressive activity of the methylated DNA-binding protein MeCP2 is lost. As discussed in chapter 1, MeCP2 mutations could lead to altered cell genesis during cortical development, as MeCP2 represses gliogenic genes during neurogenesis. Mutations in another methyl-DNA binding protein gene encoding MBD-1 leads to impaired neurogenesis of neural precursors (Zhao et al., 2003; Li et al., 2008), and are associated with autism-like behaviors (Li et al, 2005; Allan et al., 2008).

Similarly, other types of disorders displaying mental retardation may be caused by perturbations in early neural cell genesis. One example is Thanotophoric Dysplasia type II, caused by activating FGFR3 mutations (Tavormina et al., 1995; Chen et al., 2001), where fetuses and newborns display macrocephaly, seizures, as well as limitations in motor and intellectual development (Tavormina et al., 2001; reviewed in Hevner et al., 2005; discussed in Thomson et al., 2007). A conditional mouse model of this disease shows increased proliferation of neural precursors between E11-E14, as well as a profound decrease in apoptosis (Inglis-Broadgate et al., 2005), which most likely explains the bigger brain phenotype observed.
Thus, we propose that genetic perturbations such as those seen in Noonan Syndrome, or perhaps in even more commonly studied autism spectrum disorders, might first impact on cell genesis during embryonic development, and that perturbations in the initial establishment of the nervous system may then alter many aspects of neural development, resulting in impaired circuitry and cognitive dysfunction. Because SHP-2 and other components of the Ras signaling pathway play multiple functions during cortical development, it is difficult to conclude that perturbations in cell genesis lead to mental retardation. For example, one study suggests that the hippocampal-related deficits in a mouse model of neurofibromatosis are reversible, and can be treated in adulthood mice with lovastatin (Li et al., 2005). Although the authors of this study suggest that the neurological impact of NF1 mutations is reversible, compensatory mechanisms could also play a role here. Mouse models where these genes are activated in precursors during specific periods of cortical development remain to be investigated, but could be used as a tool to address these questions. The developmental cell genesis model that we and a few others have proposed does not preclude the importance of later perturbations that might occur in neuronal circuits during synapse formation and plasticity as a direct consequence of activated SHP-2 or other disorder-associated mutations. It simply argues that earlier impairments such as altered cell genesis could have more widespread effects because they occur so early during development.

5.3 Lfc and Tctex-1 regulate neurogenesis from radial precursors

The cellular mechanisms that allow the embryonic neural environment to determine the self-renewal versus differentiation of neural precursors remain a key question in neural development. While our understanding of molecular mechanisms regulating the timing and extent of neurogenesis and gliogenesis during cortical development has grown in the last decade, the mechanisms that regulate the switch from symmetric to asymmetric precursor cell division during neurogenesis have only started to be addressed. One proposed mechanism is the regulation of mitotic spindle and cleavage plane orientation in dividing radial precursors, which changes over the course of cortical development and correlates with the cell-fate decisions of daughter cells (Chenn & McConnell, 1995; Haydar et al., 2003). Although much work has been done in model systems such as *D. melanogaster* and *C. elegans* (reviewed in Gönczy, 2008; Knoblich, 2008) to support such a spindle orientation
model, only a few polarity genes have been shown to control vertebrate precursor spindle orientation during neurogenesis, including LGN (Morin et al., 2007; Konno et al., 2008), Nde1 (Feng et al., 2004) LIS1 (Tsai et al., 2005; Yingling et al., 2008; Pawlisz et al., 2008), Par proteins (Costa et al., 2007) and signaling proteins such as AGS3 and G proteins (Sanada & Tsai, 2005), and doublecortin-like kinase (Shu et al., 2006). In the second part of my thesis, I offer support for this model, implicating the Rho-GEF Lfc and Tctex-1 in a novel pathway that regulates mitotic spindle orientation in vertebrates.

5.3.1 Summary of Major Conclusions

The data presented in Chapter 4 support four major conclusions. First, our data demonstrate that Lfc is essential for the normal genesis of neurons from embryonic radial precursors. Lfc knockdown in precursors in culture or in vivo blocks neurogenesis and maintains cells as cycling radial precursors. In contrast, it has no effect on astrocyte formation. Second, we show that a negative regulator of Lfc, Tctex-1, functions in an opposite manner, and normally serves to inhibit neurogenesis. Genetic silencing of Tctex-1 either in culture or in vivo increases neurogenesis and decreases the proportion of cycling radial precursors. Third, our data indicate that Lfc and Tctex-1 are part of a common genetic pathway, where Tctex-1 normally functions by inhibiting the proneurogenic effects of Lfc. Finally, we show that Lfc regulates mitotic spindle orientation in dividing apical precursors, thereby providing a mechanism to explain its ability to regulate neurogenesis. Specifically, Lfc knockdown enhances vertical, potentially symmetrical apical divisions, thereby increasing the proportion of dividing radial precursors, and decreasing the proportion of neurogenic basal progenitors and newly-born neurons. Together, our findings support a model where the number of asymmetric, neurogenic versus symmetric, self-renewing radial precursor divisions is regulated by the interplay between the microtubule-interacting Lfc, and its negative regulator Tctex-1.

5.3.2 Mechanisms for the neurogenic function of Lfc

One key issue arising from these studies is the mechanism whereby Lfc regulates neurogenesis. We proposed that Lfc promotes neurogenesis by regulating mitotic spindle orientation, a concept that is still controversial in the field. Lfc is associated with
microtubules in the mitotic spindle of non-neural cells (Benais-Pont et al., 2003; Bakal et al., 2005), and, as shown here, in radial precursors. In non-neural cells, Lfc is required for the formation of the mitotic spindle during prophase/metaphase, for proper tethering of the astral microtubules to the cortex and for proper orientation of the mitotic spindle within the cell (Bakal et al., 2005). Here, we show that genetic knockdown of Lfc results in increased vertical divisions of apical, potentially radial, precursors. Previous work has shown that changes from vertical to horizontal planes of apical precursor division correlate with changes in neurogenesis and in cell fate determination in the mammalian cortex (Chenn & McConnell, 1995; Haydar et al., 2003; Feng et al., 2004; Sanada & Tsai, 2005; Shu et al., 2006; Yingling et al., 2008). In model organisms such as drosophila, the plane of precursor cell divisions determines proper segregation of cell-fate determinants, thereby dictating the phenotype of the daughter cells. For example, Pins, Gai and MUD form a complex that regulates mitotic spindle orientation in mushroom body and larval neuroblasts, and mutations in these genes cause defects in spindle orientation and cell fate determination, similar to the phenotypes documented here (reviewed in Gonzalez et al., 2008; Zhong & Chia, 2008). However, the importance of cleavage plane orientation to symmetric versus asymmetric divisions and cell fate in the mammalian cortex is still an open question, with data arguing both for (Feng et al., 2004; Sanada & Tsai, 2005; Shu et al., 2006; Yingling et al., 2008) and against such a mechanism (Konno et al., 2008; Noctor et al., 2008; reviewed in Zhong & Chia, 2008). Nonetheless, our finding that Lfc knockdown causes increased vertical apical precursor divisions coincident with decreased neurogenesis supports the idea that cleavage plane is also important for mammalian neural precursors, and identifies a potential mechanism for Lfc's neurogenic actions.

How then does Lfc regulate spindle orientation in radial precursors? Correct positioning and morphological changes of the mitotic spindle are cellular events that require the regulated interaction of astral microtubules and cortical F-actin, via which the centrosomal spindle poles are tethered to the cell cortex (Woolner et al., 2008). In neural precursors, as in many other systems, mitotic spindle orientation is determined by the end of metaphase, where the spindle undergoes rapid oscillations and rotations during early metaphase before taking its final orientation (Chenn & McConnell, 1995; Haydar et al., 2003). As discussed earlier, a few centrosomal proteins have been shown to regulate mitotic
spindle orientation and cell fate outcomes in dividing neural precursors (Feng et al., 2004; Tsai et al., 2005; Shu et al., 2006; Yingling et al., 2008; Pawlisz et al., 2008). How might Lfc participate in a pathway that regulates mitotic spindle orientation and fate determination? It likely does so by localizing to spindle and astral microtubules, as we and others have shown, and activating RhoA at these sites, or perhaps even at spindle poles or cortical attachment sites, to regulate F-actin dependent regulation of mitotic spindle orientation, which would presumably also be Rho-dependent. Lfc is a RhoA-specific guanine exchange factor (GEF) (Glaven et al., 1996; Ren et al., 1998; Glaven et al., 1999) and the regulation of mitotic spindle assembly and orientation by Lfc in nonneural cells is Rho signaling-dependent (Bakal et al., 2005). Additionally, LPA-mediated formation of stress fibers in Rat2 cells is dependent on Lfc-Rho signaling (Greeve et al., submitted a). In this regard, RhoA and its family member cdc42 are highly expressed in the VZ/SVZ during embryonic telecephalic development (Olenik et al., 1999; Nguyen et al., 2006), coinciding with Lfc expression. While cdc42 has recently been shown to regulate radial precursor cell polarity and fate without affecting mitotic spindle orientation (Cappello et al., 2006), Rho has been implicated in the regulation of mitotic spindle orientation in the chick neuroepithelium (Rosko et al., 2006). Although RhoA is one attractive downstream target of Lfc, the Rho-GEF also interacts with other partners such as the adherens junction-associated protein cingulin (Aijax et al., 2005), which may also be important as Lfc regulators or effectors in neural precursors. Lfc also interacts with neurabin and spinophilin in postmitotic neurons, where it regulates dendritic morphology (Ryan et al., 2005). While all known functions of Lfc are mediated by Rho, we were not able to rescue the Lfc deficiency by overexpressing an activated RhoA or to mimick these effects by transfecting precursors with a GEF-deficient Lfc or dominant-negative form of RhoA. These studies were impeded by technical problems in the case of the RhoA expression vectors, while overexpressing wildtype or GEF-dead Lfc led to cytoskeletal abnormalities and massive apoptosis of precursors, probably because of ectopic interactions and Rho-activation throughout the cell. It has been historically difficult to determine the role of Rho in neural precursors (Rosko et al., 2006), probably because it has multiple functions. We propose that Lfc regulates subcellular-specific RhoA activation, including at sites where microtubule depolymerization and actin remodeling are coordinated to generate positioning forces on mitotic spindles.
5.3.3 A novel Rho-regulatory pathway regulates neurogenesis

The genetic data presented here also indicate that the Lfc interacting partner, Tctex-1, functions upstream of Lfc in radial precursors. In nonneural cells, Tctex-1 and Lfc directly interact, and Tctex-1 functions to negatively regulate Lfc during LPA-mediated stress fiber formation (Greeve et al., submitted a). Interestingly, Tctex-1 has previously been detected at mitotic spindles in fibroblasts (Tai et al., 1998) and has been implicated in dynein-dependent transport of polarized molecules in epithelial cells (Tai et al. 1999), raising the possibility that Tctex-1 might segregate fate determinants in neural precursor cells (Dedesma et al., 2006). Tctex-1 also has dynein-independent roles, one of which is to function as a nonreceptor activator of heterotrimeric G-protein signaling (Tctex-1 is also known as AGS2; Takesono et al., 1999; reviewed in Lanier, 2004). Intriguingly, a recent study showed that AGS3, another nonreceptor activator of G-protein signaling (and a mammalian Pins homologue) and Gβγ signaling regulate mitotic spindle orientation and neurogenesis in cortical precursors (Sanada and Tsai, 2005). In this regard, Tctex-1 and Lfc are also downstream of G-protein signaling, at least during stress fiber formation (Greeve et al., submitted a), suggesting that Gβγ, Tctex-1 and AGS3 might somehow converge to regulate the proneurogenic actions of Lfc. Such genetic and biochemical interactions remain to be shown in neural precursors.

While our data show that silencing of Tctex-1 enhanced neurogenesis, one unexpected observation was that many of these neurons remained within the VZ/SVZ and, of those that migrated, many did not end up in their appropriate layers. There are many potential explanations for this deficit in migration, but one interesting possibility is that silencing of Tctex-1 led to activation of RhoA in newly-born neurons, which inhibits their migration out of the VZ/SVZ following cell cycle exit. (Kholmanskikh et al., 2003; Hand et al., 2005; Nguyen et al. 2006). Another possibility is that Tctex-1 knockdown coincidently increased the number of newly-born neurons and decreased the number of radial precursors, which these neurons use as a scaffold for directed migration.

Altogether, evidence suggests that Tctex-1 and Lfc may be part of a genetic pathway that coordinates microtubule and Rho-dependent actin cytoskeletal rearrangements at defined subcellular locations. It remains to be determined whether the role of Tctex-1 in this process is dynein-dependent or –independent. Interestingly, cultured Lfc+/− or Lfc−/− E12 precursors...
do not show significant defects in proliferation or neurogenesis. This suggests that other Dbl-related proteins such as Lsc or Lbc or other Rho-regulators may compensate for loss of Lfc expression in the Lfc knockouts, whereas compensatory mechanisms would likely not be present in our acute knockdown approach. We are currently investigating the phenotypes of conditional Lfc knockout precursors transfected with cre recombinase.

5.3.4 Alternative mechanisms for the regulation of neurogenesis by Lfc and Tctex-1

Another model for the asymmetric inheritance of fate determinants during neurogenesis has been proposed, and involves a mitotic spindle-independent process. In this process, apical membrane components of dividing precursors become asymmetrically distributed due to a regulated loss of a small prominin-rich portion of the membrane (Kosodo et al., 2004; Marzesko et al., 2005). As such, apical fate determinants are inherited asymmetrically following a vertical or perpendicular division due to a regulated loss of prominin-rich portion of the membrane (reviewed in Huttner & Kosodo, 2005). While the importance of such a model remains to be determined, the regulation of symmetric and asymmetric divisions in mammalian neural stem cells probably involves more than controlled mitotic spindle orientation. Furthermore, regulation of mitotic spindle orientation in mammalian stem cells most likely involves multiple players, some of which are not evolutionarily-conserved, as illustrated by our study. Recent studies (Fish et al., 2006), including the one presented here, suggest that small changes in mitotic spindle orientation can alter cell fates. As such, the conventional measurements of mitotic spindle orientation defining symmetric versus asymmetric divisions may need to be revised, as we and others (Fish et al., 2006) have done. This may reconcile some of the conflicting results in the literature, where, for example, Noctor and colleagues have suggested that specific types of precursors do not alter their modes of division throughout corticogenesis (Noctor et al., 2008).

Our preliminary results also demonstrate that Tctex-1 knockdown affects mitotic spindle orientation establishment and maintenance, leading to decreased proportions of potentially vertical (symmetric) divisions. Although Lfc and Tctex1 appear to play a role in radial to basal precursor transitions by regulating mitotic spindle orientation, we cannot exclude that they may also regulate neuronal fate determination via other mechanisms. In
Rat2 fibroblasts, Lfc and Tctex1 are known to interact on microtubules at sites of stress fiber formation (Greeves et al, submitted). It is therefore possible that Lfc and Tctex-1 play additional roles at the level of cytoskeletal rearrangement required for migration and differentiation for neuronal precursors and for migration of newly-born neurons. Such is the case for other multifunctional proteins such as LIS1 during cortical development, where LIS1 is required for proper mitotic spindle orientation of precursors, interKininetic nuclear migration, as well as neuronal migration (Yingling et al., 2008; Gambello et al., 2003; Tsai et al., 2005; Tsai et al., 2007). Another possibility is that Lfc and Tctex-1 play roles in cell cycle regulation. RhoGTPases are known to regulate mitosis (reviewed in Narumiya & Yasuda, 2006) and, as a Rho-GEF, it is likely that Lfc could also regulate this process. Thus, Tctex-1 and Lfc may regulate Rho at specific subcellular locations and mediate various aspects of neuronal fate determination and differentiation. Conversely, Tctex-1 and Lfc may also play independent roles in these events. For example, Tctex-1 may play dynein-independent roles as an AGS protein in this context, or dynein-dependent roles. On that note, dynein has been implicated in the regulation of many aspects of neurogenesis, including precursor divisions and neuronal migration (Tsai et al., 2007; Yingling et al., 2008).

Other related Rho- and actin-dependent processes, independent of mitotic spindle orientation, could be regulated by Lfc and Tctex-1 during cortical precursor development and neurogenesis. For example, trafficking and distribution of fate determinants is an actin-dependent process, where disruption of actin polymerization leads to defects in cytokinesis and dysregulation of asymmetric localization of EGFR in mammalian cortical precursors (Sun et al., 2005) and fate determinants numb and prospero in drosophila (Knoblich et al., 1997; Knoblich, 2008). Additionally, the expression or localization of polarity proteins such as cadherins, which are required for radial precursor maintenance and ventricular zone integrity, may also be impeded by a decrease in Lfc activity. For example, N-Cadherin protein levels and cell-cell contacts are regulated by Rho and cdc42 in P19 embryonal stem cell during neurodetermination (Laplante et al., 2004), and by cdc42 in neural precursors (Cappello et al., 2006). This may in part explain the interesting observation that many Pax6-positive Lfc shRNA-transfected cells are aberrantly found in the SVZ rather than VZ in our electroporation experiments, suggesting that they have lost their ventricular zone attachment. Although this has not yet been tested, it will be done by assessing the expression of polarity...
proteins in our electroporation experiments, and by confocal reconstruction of the mislocalized cells to determine whether these cells are still connected to the ventricular surface by endfeet.

5.3.4 Alternative cell fates

An obvious question regards the alternate fate of the transfected cells which accumulate below the cortical mantle. In the LfcsRNA-transfected cortices, a large proportion of transfected cells accumulate in the SVZ, consistent with the increase in mislocalized Pax6-positive transfected cells. Interestingly, at postnatal day 3, many long processes that reach the pial surface stem from these cells, suggesting that they are a remnant of radial precursors or neurons that are extending a leading process without migrating. Our data suggest that the majority of these precursors do not differentiate into neurons or glial cells by E17-18 or postnatal day 3. Although we have tested the expression of many antigenic markers, we have found none that labels all of these cells, and the majority of these accumulated cells do not co-express pax6, nestin, blbp, doublecortin, βIII tubulin, HuD or GFAP. Although an increase in proliferating radial precursors is detected early on, by four days post-electroporation and postnatal day three the majority of the remaining cells are not Ki67+ve, suggesting that they have exited the cell cycle. The increase in Pax6+ve cells in the SVZ at early stages suggests that they may have been radial precursors destined to generate neurons, but have lost their apical attachments and polarity. When this occurs in other examples such as cdc42 deficiency, these cells adopt characteristics of basal precursors and generate neurons, with no significant effects on total neuronal output (Cappello et al., 2006). This does not occur here, suggesting that other mechanisms inhibit this alternate fate. One possibility is that these cells remain stuck as early “messsed up” neuronal precursors that no longer respond to their environmental cues and stop proliferating without differentiating into neuronal cells. One way to determine if this is the case would be to dissect and culture these transfected cells in a mitogenic or neurogenic environment, and see whether they ultimately generate neurons. Due to the increase in proliferation at early stages, one might expect that more neurons would ultimately be generated from such precursors.
5.3.5 What are the extracellular signals that regulate Tctex-1 and Lfc function?

The cellular mechanisms that allow the embryonic neural environment to determine the self-renewal versus differentiation of neural precursors remains a key question in neural development. Our findings, together with previous work in nonneural cells, imply that one way the environment might control self-renewal versus neurogenesis in the embryonic cortex is via G-protein coupled receptors (GPCRs) and Tctex-1/ Lfc. In this regard, many G-protein coupled neurotransmitter receptors regulate neural precursor proliferation and neurogenesis (reviewed in Cameron et al., 1998; Nguyen et al., 2001). Other possible candidates include the LPA receptor vzg1 which is highly expressed in cortical precursors (Hecht et al., 1996), and GPR56. Human mutations in GPR56, a GPCR expressed in the rodent ventricular zone at E12-14, cause microcephaly and cortical lamination defects, suggesting that GPR56 may well regulate neurogenesis (Piao et al., 2004; Ke et al., 2008). Studies addressing the effects of LPA on cortical precursors have recently implicated this GPCR ligand in the regulation of self-renewal and neurogenesis. Dottori and colleagues demonstrated the inhibitory effect of LPA on neuronal differentiation using human embryonic stem cell-derived neural precursors in culture, with no effects on proliferation, survival or astrocyte formation (Dottori et al., 2008). Interestingly, Estivill-Torrus and colleagues observed a reduction of VZ thickness as well as decreased proportions of proliferating and Pax6+ve precursors with a concomitant increase in ectopically-localized neurons in the developing cortex of LPA receptor 1-deficient mouse embryos in vivo. Consistently, increases in non-vertical divisions were observed in the lpa1-deficient ventricular zone when compared to wild-type counterparts (Estivill-Torrus et al., 2008), suggesting that LPA signaling promotes symmetric divisions in a fashion similar to tctex-1. In addition to these fate defects, LPA1-deficient cortices displayed increased cell death suggesting that GPCR signaling may regulate multiple aspects of neural precursors via different cellular targets, including tctex-1. One of the important hypotheses stemming from our and similar studies is that alteration in embryonic precursor divisions can lead to changes in the population of remaining adult precursors. Consistently, in a follow up study of adult hippocampal neurogenesis in the same LPA1-deficient mouse model, Matas-Rico and colleagues observed a reduction in adult neural precursors, leading to defects in neuronal differentiation (Matas-Rico et al., 2008). We are currently investigating
whether our manipulations of Lfc and Tctex-1 levels have similar effects on adult neural stem cell niches.

5.4 General discussion

5.4.1 Link between growth factor signaling and mitotic spindle orientation?

It remains unclear how growth factors regulate proliferation and differentiation of neural precursors, or how growth factors regulate neuroepithelial, radial and basal precursor transitions. More importantly, do growth factors such as neurotrophins and PDGF promote neurogenesis by acting prior to or following precursor divisions? Do they regulate the types of division or regulate the fate of the resulting progeny? This has not been clearly addressed or distinguished, and may be a novel avenue of research to pursue.

Could growth factors regulate mitotic spindle orientation and divisions in cortical precursors? Alternatively, does mitotic spindle orientation regulate the distribution of fate determinants such as receptors, membrane-associated proteins, signaling proteins and transcription factors during cortical precursor divisions? In response to the first scenario, MEK-ERK signaling has previously been linked to mitotic spindle orientation determination in other cell types, more specifically in maturing rodent oocytes. Phosphorylated MEK is detected at spindle poles during meiosis, and MEK signaling regulates microtubule organization, spindle pole tethering and asymmetric division in this context (Horne & Guadagno, 2002; Yu et al., 2007; Sun et al., 2008). Interestingly, in adult neural stem cells, growth factor receptors such as PDGFR and EGFR have been detected at the mitotic spindle during mitosis (Danilov et al., 2008), and some FGFR2 isoforms localize to the nucleus (Powell et al., 1991b), although the nuclear function of FGFR2 is unclear. How and why these RTKs may translocate from cell surface membranes to the nucleus or mitotic spindle apparatus during mitosis is an interesting and open question. Is this a means of segregating the receptors between progeny during division, or do these receptors, perhaps following their activation at the membrane, translocate and signal at microtubules? Interestingly, Tctex-1 interacts with the cytoplasmic tail of a number of growth factor receptors known to regulate embryonic cortical precursor proliferation, survival, and differentiation, including Trk neurotrophin receptors (Yano et al., 2001; Bartkowska et al., 2007) and BMP receptor type II (Machado et al., 2003; Li et al., 1998; Mabie et al., 1999; Gross et al., 1996; Gomes et al.,
suggesting that it could either be involved in dynein-dependent trafficking of these receptors on microtubules or dynein-independent regulation of signaling events downstream of these receptors. Thus, Tctex-1 and Lfc are positioned to integrate multiple environmental signals and to play a key role in determining self-renewal versus neurogenesis in the developing cortex.

While more studies are required to confirm the presence and roles of these molecules at mitotic spindles in embryonic cortical precursors, this perspective begs the question of whether MEK-ERK and Rho signaling interact to regulate neural precursor fate at this level? In nonneural cells, ERK-dependent phosphorylation of Lfc at T678 (Figure 1.6) enhances the GEF activity of Lfc towards Rho (Fujishiro et al., 2008). Could the integration of the two neurogenic signaling pathways studied in this thesis be a relevant mechanism for the regulation of neurogenesis? An interesting hypothesis is that local activation of Lfc by RTK signaling, along with its release from negative regulators at microtubules could mediate its relocalization to spindle poles where it can activate Rho and regulate mitotic spindle orientation during metaphase. Concomitant G-protein activation by GPCR-dependent or independent signaling would potentially be required for proper regulation of this process. Due to limitations relating to cell death caused by overexpression of an Lfc cDNA, it was not possible to monitor Lfc dynamics in live cell imaging following treatments with various growth factors or pharmacological inhibitors prior to and during mitosis to address this issue. However, this may be possible if one were to design a fluorescent protein-tagged Lfc knockin mouse model.

In D. melanogaster and C. elegans models, frizzled and Wnt/β-catenin as well as receptor independent G-protein signaling regulate mitotic spindle positioning and orientation (Bellaiche et al., 2001; Walston et al., 2004; reviewed in Gönczy, 2008). This leads to the appropriate segregation of polarized fate determinants in daughter cells. In mammalian neural stem cells, little is known about the molecular mechanisms regulating the asymmetrical segregation of fate determinants during neurogenic divisions. Are important growth factor and cytokine receptors evenly distributed or polarized? For example, in this respect, the apical vs basal inheritance of receptors or signaling molecules could play a role in the fate determination of daughter cells following mitosis. Unfortunately, until recently, subcellular immunolocalization of these molecules in radial precursors has been limited.
However, as discussed previously, a few studies suggest that growth factor receptors such as EGFR are asymmetrically distributed in apical precursors, providing one mechanism by which signaling and ensuing adopted fates can differ between two daughter cells.

The cellular and molecular mechanisms that allow the embryonic neural environment to determine the self-renewal versus differentiation of neural precursors remain a key question in neural development. The findings from both studies presented in this thesis and previous work in many other systems imply that one way the environment might control fate determination in the embryonic cortex is via the regulation of polarity proteins and the integration of proliferative, neurogenic and gliogenic signaling and transcriptional cascades that are regulated by switch molecules such as SHP-2.
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