# Disturbances in progenitor cell placement, proliferation, and apoptosis modify neocortical development in the tish neurological mutant

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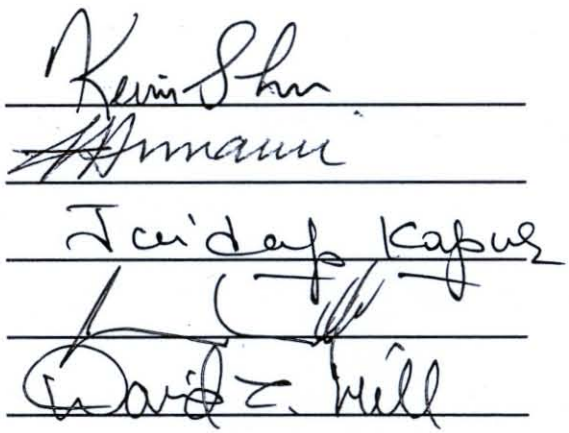

# Abstract

Cortical malformations are commonly associated with intractable epilepsy and other developmental disorders. In one such malformation, subcortical band heterotopia (SBH), large numbers of cortical neurons and glia are improperly located in a discreet band in the subcortical white matter. While theories focusing on defective neuronal migration have yielded causative genes and pathogenic mechanisms for certain human cases of this disorder, others still exist for which no causative genetic or cellular disturbances can be identified, leaving open the possibility for alternative mechanisms of SBH formation.

The tish rat is spontaneously occurring genetic model of SBH in which the malformation is inherited in an autosomal recessive manner. A unique feature of developing tish$^{-/-}$ neocortex, which has not been reported in other animal models of SBH, is the presence of an abnormally located (heterotopic) band of proliferating cells in the intermediate zone and cortical plate in addition to the normally positioned progenitors in the ventricular and subventricular zones.

The guiding hypothesis of this thesis is that heterotopic proliferative cells contribute to the formation of the tish$^{-/-}$ SBH. The experiments presented herein characterize the identity and cell cycle kinetics of these heterotopic proliferating cells and investigate the cellular mechanism underlying their mislocalization. Overall, the results demonstrate that the tish mutation disrupts the position and cell cycle kinetics of progenitors of the radial glial and intermediate progenitor lineages in the developing neocortex. This mislocalization is not associated with adherens junction breakdown or

loss of radial glial polarity in the ventricular zone. The mislocalized progenitors in tish$^{-/-}$ neocortex do not appear to maintain contact with the ventricular surface. Therefore, the affected progenitor cells may be unable to exit the cell cycle once they initiate migration, which leads to the seeding of a heterotopic proliferative zone upon their arrival in the IZ/CP. Taken together, these findings define a form of developmental error contributing to SBH formation that differs fundamentally from a primary error in neuronal migration.

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# Acknowledgements

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# Chapter 1

## Introduction

Normal development of the mammalian neocortex is a complex process of precisely timed molecular and cellular events, the details of which continue to be elucidated and refined. The requirement for such intricate control of developmental events becomes apparent when one considers the scope of the problem at hand. Neurons destined for the cortex are born in specific sites bordering the cerebral ventricles, after which they must embark on a sometimes lengthy and convoluted migration to their future place of residence in the cortex. Upon arrival, neurons must orient appropriately in their designated layer and establish proper afferent and efferent connections. Considering this sequence of events, it is not surprising that developmental errors are relatively common, resulting in an incidence of cortical malformation in humans of at least 1% (Meencke and Veith, 1992). Interestingly, some manner of cortical malformation is present in approximately 14% of patients with epilepsy and approximately 40% of patients with medically-intractable epilepsy (Hardiman et al., 1988; Farrell et al., 1992; Meencke and Veith, 1992).

One of the major types of cortical malformations, lissencephaly, occurs at a frequency of at least 1 in 100,000 live births, with the frequency likely closer to 1:13,000 or 1:20,000 (Dobyns et al., 1993). Lissencephaly is a malformation in which the cortical surface is characterized by agyria (lack of gyri or sulci), pachygyria (broadened gyri), or a mix of the two. Patients with lissencephaly exhibit severe mental retardation and other neurological abnormalities, including epilepsy typified by seizures of a myoclonic, tonic, or tonic-clonic type (Dobyns et al., 1993). Related to lissencephaly is a condition called subcortical band heterotopia (SBH) in which post-mitotic neurons collect in a heterotopic band of cells located within the subcortical white matter. Patients with SBH can display

a variable degree of cognitive impairment, with or without seizures or more severe epilepsy (des Portes et al., 1998a). While some authors have suggested a role for disordered cellular proliferation or apoptosis in lissencephaly/SBH (Rorke, 1994; Eksioglu et al., 1996; Lee et al., 1998a), this spectrum of malformations traditionally has been classified as a neuronal migration disorder. Despite this classification and the supporting causative genes and pathogenic mechanisms that have arisen from it, there exist some human cases of classical lissencephaly/SBH for which no causative gene or mechanism has been identified, leaving open the possibility for alternative hypotheses of lissencephaly/SBH pathology (Leventer, 2005; Delatycki and Leventer, 2009).

## Clinical features and significance of lissencephaly/SBH

### Clinical characteristics and syndromes

Patients with classical lissencephaly, also called type I lissencephaly, show evidence of agyria or pachygyria as a central feature of this disorder. The cerebral cortex of these individuals appears abnormally thick and is organized into roughly four layers. Accompanying this structural malformation is a host of neurological abnormalities. A large majority of patients experience seizures of a tonic, clonic, or tonic-clonic type within the first year of life. Frequently, these patients suffer from severe mental retardation. Other neurological abnormalities can include early hypotonia with later spastic quadriplegia, and opisthotonos, an abnormal posturing characterized by rigidity and severe arching of the back. In addition, infants with lissencephaly are often small for gestational age, experiencing failure to thrive, with polyhydramnios (excessive amniotic fluid surrounding the fetus) as a non-specific finding during gestation. Some children

may be microcephalic at birth, but most will develop microcephaly within the first year of life (Dobyns et al., 1993).

Classical lissencephaly can occur as an isolated birth defect, termed isolated lissencephaly sequence (ILS), which can be X-linked (XLIS), or as a component of a congenital malformation syndrome called Miller-Dieker syndrome (MDS). MDS consists of severe lissencephaly with facial abnormalities such as a prominent forehead, bitemporal hollowing, short nose with upturned nares, small jaw, and a prominent upper lip with thin or downturned vermilion border (Dobyns et al., 1993; Kato and Dobyns, 2003). ILS differs in that the lissencephaly is typically less severe, and facial appearance tends to be normal (Dobyns et al., 1993). The neocortical phenotype in XLIS resembles that in ILS; however, lissencephaly in XLIS affected families manifests in males whereas females demonstrate subcortical band heterotopia (Ross et al., 1997).

Patients affected with subcortical band heterotopia (SBH) demonstrate bilateral bands of heterotopic gray matter located within the subcortical white matter. These heterotopia vary in size across patients and may extend from the frontal to occipital lobes or may restrict themselves to more discreet fronto-parietal or parieto-occipital distributions. The overlying gyri vary between a normal appearance and one of mild pachygyria. Children manifesting SBH, the overwhelming majority of whom are female, can suffer from mental retardation and epilepsy. Seizure types can include partial and generalized tonic or tonic-clonic, partial complex, atonic, or atypical absence. Intelligence ranges from normal (approximately 18% of patients) to mental retardation of borderline (~10%), mild (~32%), moderate (~25%), or severe (~16%) degree (Dobyns et al., 1996). The severity of epilepsy and mental retardation in these patients has been

shown to correlate directly with the relative thickness of the heterotopic band as measured by MRI (Barkovich et al., 1994). Other deficits include delays in speech development and in the onset of walking (des Portes et al., 1998a).

Pathogenesis of lissencephaly/SBH: the Lis1 and DCX genes

Presently, the genetic basis of approximately 10-20% of classical lissencephaly and SBH cases remains unknown (Delatycki and Leventer, 2009). Two genes, Lis1 and doublecortin (DCX) are responsible for most of these cases, although other genes such as aristaless-related (Arx), α-tubulin (TUBA3), reelin (RELN), very-low density lipoprotein receptor (VLDLR), and ApoE receptor 2 (ApoER2) have been shown to cause classical lissencephaly (Reiner et al., 1993; Hirotsune et al., 1995; des Portes et al., 1998b; Trommsdorff et al., 1999; Kitamura et al., 2002; Keays et al., 2007). Lis1 was isolated in 1993 as the gene deleted from autosomal chromosome 17p13.3 in classical lissencephaly patients with Miller-Dieker syndrome (Reiner et al., 1993). It has since been shown to be a causative gene defect in ILS as well (Ross and Walsh, 2001). While haploinsufficiency due to gene deletion has typically been observed in patients, missense mutations in Lis1 have been shown to result in milder phenotypes than truncation/deletion mutations (Cardoso et al., 2000). In addition, while defects in radial migration of neurons resulting from haploinsufficiency of Lis1 have traditionally been assumed to underlie the pathology of MDS/ILS, Lis1 has also been shown to be necessary for non-radial migration of interneurons in mice and in patients with MDS (McManus et al., 2004; Pancoast et al., 2005).

Biochemical studies of Lis1 have identified three distinct regions within the protein sequence. The N-terminal amino acids 1-39 contain the Lis1-homology domain

(LisH), a novel motif which has been implicated in protein homodimerization or regulation of microtubule stability either directly or via interaction with cytoplasmic dynein (Emes and Ponting, 2001). Amino acids 40-85 delineate a coiled-coil region, which, in conjunction with the LisH domain, contributes to homodimerization of Lis1 (Kim et al., 2004), though presence of the coiled-coil region is not critical for this effect (Tai et al., 2002). Lastly, residues 96-410 form a β-propeller structure containing seven C-terminal WD40 repeats, which are thought to mediate the interactions of Lis1 with intracellular targets (Reiner et al., 1993).

Functional studies of Lis1 in multiple model systems have demonstrated that Lis1 acts as a non-catalytic subunit of platelet activating factor acetylhydrolase (PAFAH) and as an adapter for the cytoplasmic dynein complex (Hattori et al., 1994; Emes and Ponting, 2001; Tarricone et al., 2004). While the functions of the PAFAH complex during brain development are unclear, it has been shown to influence differentiation and migration, particularly through interactions with various elements of the reelin signaling pathway (Kornecki and Ehrlich, 1988; Prescott et al., 2000; Tokuoka et al., 2003; Zhang et al., 2007; Zhang et al., 2009). As a component of the cytoplasmic dynein complex, Lis1 plays important roles in mitotic progression and cell division, actin and microtubule reorganization, centrosome movement, and nuclear translocation in migrating cells, and protein-protein interactions between components of the cytoplasmic dynein motor complex as well as other important developmental regulators such as 14-3-3ε, CLIP-170, CLIP-115, Dab1, and DCX (Faulkner et al., 2000; Feng et al., 2000; Kitagawa et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000; Smith et al., 2000; Aumais et al., 2001; Cahana et al., 2001; Coquelle et al., 2002; Tai et al., 2002; Assadi et al., 2003;

Dujardin et al., 2003; Toyo-oka et al., 2003; Lansbergen et al., 2004; Shu et al., 2004; Tanaka et al., 2004b; Sasaki et al., 2005; Tsai et al., 2007; Kawauchi and Hoshino, 2008; Pawlisz et al., 2008; Yingling et al., 2008).

DCX was mapped to chromosome Xq22.3-q23 in 1997 in families containing males with an X-linked variant of classical lissencephaly and females with SBH (Ross et al., 1997). Later, DCX was specifically cloned as the major causative gene in XLIS and SBH, supporting the concept that X-inactivation might lead to different phenotypes between genders and variable clinical severity within genders (des Portes et al., 1998a; des Portes et al., 1998b). Detailed sequence analysis of DCX revealed an evolutionarily conserved, novel domain within the N-terminus, named the DC domain, consisting of two 80 amino acid repeats in tandem (Sapir et al., 2000; Taylor et al., 2000). Interestingly, this DC domain was shown to play a role in microtubule binding to DCX, and mutations in the DCX gene in XLIS patients tended to cluster in patterns that defined the tandem (Sapir et al., 2000; Taylor et al., 2000), with certain mutations disrupting microtubule binding (Yoshiura et al., 2000). Functional studies have demonstrated the importance of DCX in mediating microtubule dynamics and protein-protein interactions in migrating neurons (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999; Friocourt et al., 2001; Kizhatil et al., 2002; Gdalyahu et al., 2004; Graham et al., 2004; Moores et al., 2004; Schaar et al., 2004; Tanaka et al., 2004a; Friocourt et al., 2005; Tsukada et al., 2005). Efficient regulation of microtubule dynamics, as well as the recruitment of a wide range of binding partners to microtubules, is critical for maintaining the integrity of the cytoskeleton during neuronal migration and for facilitating neuronal migration from the proliferative compartments to the cortical plate.

Independent of its appearance within the DCX protein, the DC domain has been observed in several other proteins and has been shown to mediate binding to microtubules, establishing a novel family of microtubule binding motifs. Examples of such proteins include doublecortin-like kinase (DCLK) (Burgess and Reiner, 2000) and its splice variant doublecortin-like (DCL), doublecortin-domain-containing gene (DCDC1) (Zeng et al., 2003), and doublecortin kinase-2 (DCK2) (Edelman et al., 2005). The functions of each of these proteins and their relationships to DCX continue to be elucidated, though the majority of focus has been directed toward DCLK and DCL. DCL has been shown to be important for mitotic spindle formation, radial glial proliferation, and radial process stability, and it is expressed early in corticogenesis in proliferative compartments compared with DCX, which is expressed more strongly as neurogenesis accelerates (Vreugdenhil et al., 2007; Boekhoorn et al., 2008). DCLK has been shown to be partially functionally redundant with DCX, serving important roles in processes such as hippocampal and neocortical morphogenesis, radial and tangential neuronal migration, midline decussation of fiber tracts, axonal transport of synaptic vesicle proteins, M phase progression and mitotic spindle integrity, and daughter cell fate selection (Deuel et al., 2006; Koizumi et al., 2006; Shu et al., 2006; Tanaka et al., 2006; Friocourt et al., 2007). Despite their varied effects on the cell biology of neural progenitor cells and migrating neurons, Lis1 and DCX have been shown to facilitate migration cooperatively (Figure 1-1). Both proteins interact directly, and DCX overexpression rescues deficits in migration and nucleus-centrosome coupling in $Lis1^{+/-}$ neurons (Caspi et al., 2000; Tanaka et al., 2004b). These results establish a model for neuronal migration in which microtubules couple the leading edge of the migrating neuron to the centrosome and the centrosome to

the nucleus. In this system, DCX functions as a microtubule polymerizing and reorganizing factor throughout the length of the cytoskeleton and as a targeting factor for other proteins, particularly for Lis1. At the same time, Lis1 localizes the dynein motor complex to the nuclear membrane and facilitates minus-end directed movement along perinuclear microtubules, dragging the nucleus toward the centrosome and leading edge during migration. From this perspective, deficits in Lis1 or any component of the dynein complex might lead to defective nuclear translocation and, therefore, failed migration and lissencephaly. In addition, deficits in DCX would result in failed microtubule cytoskeleton arrangement and defective localization of the dynein motor complex to perinuclear microtubules, resulting in failed migration and lissencephaly or SBH.

**Radial glial evolution from neuroepithelium to astrocyte**

Similarities and differences in molecular markers

From the beginning of CNS development, the neural tube is populated by bipolar cells with long processes that span the entire width from the ventricular surface to the pial surface. These neuroepithelial cells serve as the precursors for all future neurons and glia of the adult CNS. Proliferative capacity notwithstanding, the neuroepithelium is interesting for the transformations that its cells undergo as generation of the cortex progresses. At the outset of neurogenesis, neuroepithelial cells give rise to radial glia, which possess long radial processes and somata that reside in the ventricular zone (VZ) of the developing cortex (Rakic, 1971, , 1972). As neurogenesis comes to a close, many radial glia transform into astrocytes that populate the neocortex (Voigt, 1989). Recent investigation of this latter transition, however, utilizing pial-directed adenoviral fate

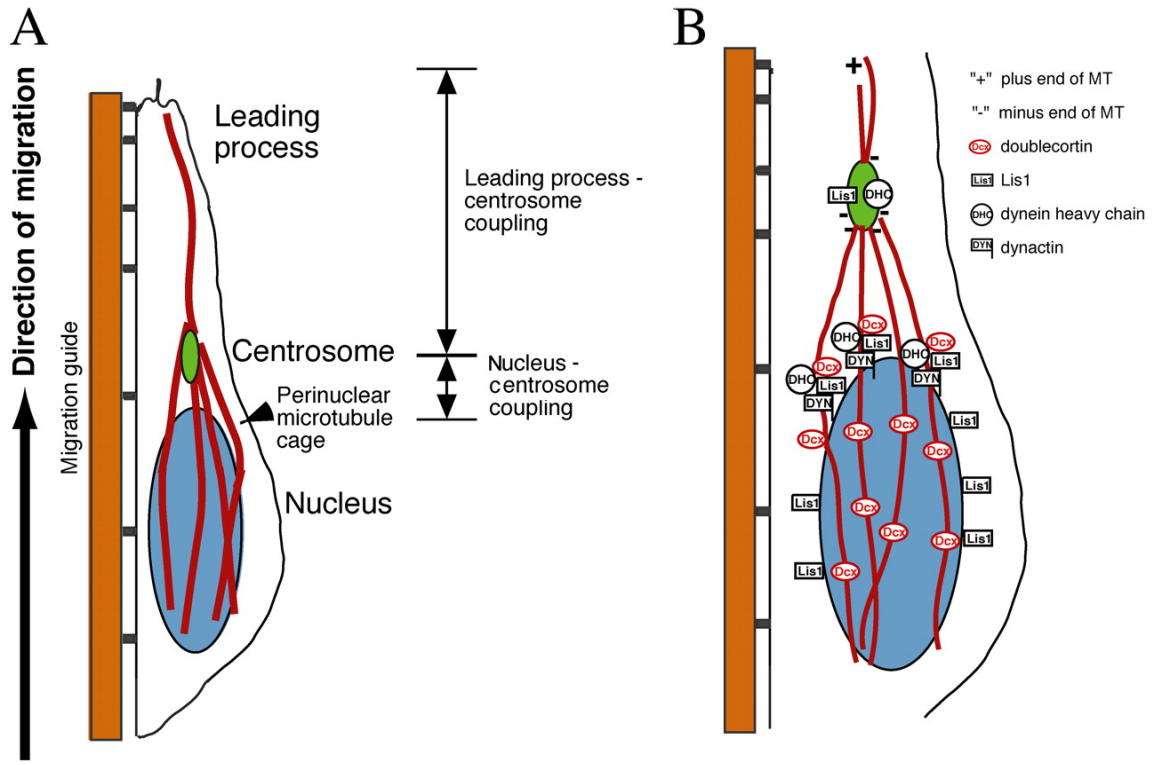
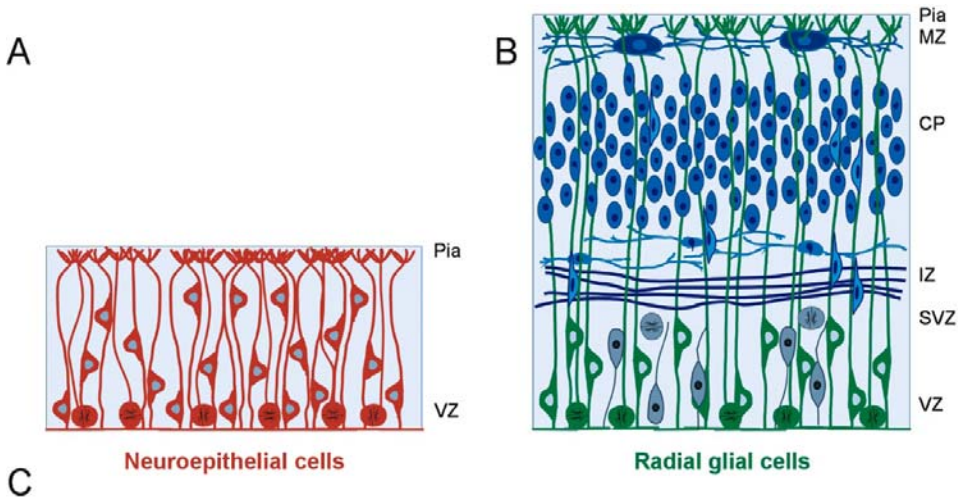



**Figure 1-1. Lis1 and DCX cooperate to control nuclear translocation in migrating neurons.** DCX localizes to microtubules of the perinuclear cage and stabilizes them while recruiting Lis1 and components of the cytoplasmic dynein motor complex to the nuclear envelope. Subsequently, the dynein motors undergo minus-end directed movement along the perinuclear microtubules, dragging the nucleus toward the centrosome. (from Tanaka et al., 2004b).

mapping strategies to label radial glia between P0 and P2 has indicated that some dorsolateral radial glia retract their processes to become $GFAP^+$ astrocytes in the postnatal subventricular zone (SVZ) and generate oligodendrocytes and olfactory bulb interneurons (Ventura and Goldman, 2007).

As would be expected, the transition of neuroepithelial cells to radial glia and the transition of radial glia to astrocytes are accompanied by alterations in the molecular markers and ultrastructural features of the cells (Figure 1-2). Most simply, changes along this lineage can be described as the acquisition of glial features and the loss of immature progenitor cell features. Though both neuroepithelial cells and radial glia are similar in their expression of the precursor cell intermediate filament, nestin (Hockfield and McKay, 1985; Lendahl et al., 1990), and its posttranslational modifications recognized by the RC1 and RC2 antibodies (Chanas-Sacre et al., 2000), several structural characteristics and markers of glia are also present. Such structural characteristics include 24-nm microtubules and 9-nm intermediate filaments within the radial fiber and cytoplasmic glycogen granules within the pial endfeet (Choi and Lapham, 1978; Bruckner and Biesold, 1981). Such molecular markers characteristic of adult CNS astrocytes include glial fibrillary acidic protein (GFAP), astrocyte-specific glutamate transporter (GLAST), brain lipid-binding protein (BLBP), and tenascin C (TN-C) (Campbell and Gotz, 2002). The timing of these changes correlates with the onset of neurogenesis in defined sets of cells (Hartfuss et al., 2001; Noctor et al., 2002). As radial glia make their final transformation into cortical astrocytes, they maintain the aforementioned astrocytic features, while losing the immature features that they held in common with neuroepithelial cells.




| Antigen/feature | Neuroepithelial cells | Radial Glia | Astrocytes |
|---|---|---|---|
| Nestin | + | + | - (reinduced in reactive astrocytes) |
| RC2 | + | + | - (reinduced in reactive astrocytes) |
| RC1 | + | + | + |
| GFAP | - | + (absent in rodents) | + |
| GLAST | - | + | + |
| BLBP | - | + | + |
| TNC | - | + | + |
| Glycogen granules | - | + | + |

**Figure 1-2. Antigenic features of neuroepithelial cells, radial glia, and astrocytes**. Evolution of the radial glial lineage in the developing neocortex can be followed using a number of molecular markers and ultrastructural characteristics. Most simply, changes along this lineage can be described as the acquisition of glial features and the loss of immature progenitor cell features (from Malatesta et al., 2003).

Interkinetic nuclear migration

An intriguing behavior shared by neuroepithelial cells and radial glia is the activity termed interkinetic nuclear migration (Misson et al., 1988). In short, the nuclei of these cells translocate within the ascending and descending process of the cell in a manner that is coordinated with cell cycle stage. During S phase, the nucleus resides in the outer portion of the ventricular zone, and, as the cell progresses through G2 to M phase, the nucleus migrates rapidly within the descending process to the ventricular surface. Once adjacent to the ventricle, the cell divides, and then the nucleus ascends through the process toward the outer portion of the ventricular zone as it undergoes G1 phase.

Given this complex behavior, one might envision the intricate cellular and molecular changes that must take place to coordinate nuclear translocation within a cell. No doubt, reorganization of cytoskeletal components with their motor proteins and associated signaling cascades would be required, creating the potential for errors that may affect cellular proliferation, migration, and positioning of daughter cells in the cortex. Despite the potential impact of errors in this process, little is known about its control on a cellular and molecular level. A study by Ueno and colleagues indicated that disruption of the cell cycle *in vivo* via administration of 5-azacytidine or cyclophosphamide results in the arrest of nuclear migration in the VZ, suggesting a direct link between cell cycle regulators and migratory machinery (Ueno et al., 2006). More recently, a study in zebrafish retina suggested that interkinetic nuclear migration regulates exposure of progenitor nuclei to gradients of proliferative versus neurogenic signals in the ventricular zone (Del Bene et al., 2008). Not only does this study ascribe a function to interkinetic

nuclear migration, but it also underscores the importance of spatial positioning in assuring a cell's exposure to appropriate environmental cues for its specification, migration, and proper functioning.

## Changing perspectives on radial glia in normal neocortical development

### From migratory scaffold to neurogenic precursors

Because of their glial nature and their unique morphology, radial glia had been viewed traditionally as support cells for neuronal migration (Rakic, 1971, , 1972, , 1978; Hatten, 1999). In favor of this static picture, it seemed teleologically wasteful for radial glia, with their long pial processes, to participate in the cell cycle, as conventional wisdom suggested that mitotic cells in this environment lost their processes and rounded up prior to undergoing mitosis (Seymour and Berry, 1975). Accordingly, radial glia acquired the limited moniker of migratory scaffold in the developing cortex, and this viewpoint persisted until new lines of evidence began to indicate a broader scope of activities for these cells.

Making use of transgenic mice expressing green fluorescent protein (GFP) under the GFAP promoter, Malatesta and colleagues used fluorescence-activated cell sorting to isolate GFP-expressing cells from embryonic day E13-E18 cortex. These cells expressed several radial glial antigens including RC2, BLBP, and GLAST. *In vitro*, the progeny of radial glia sorted during peak neurogenesis yielded pure neuronal clones, while cells sorted near the end of neurogenesis yielded astrocytes (Malatesta et al., 2000). *In vivo*, using in utero injection of GFP-expressing retrovirus into embryonic rat lateral ventricles, Noctor et al. labeled radial glial cells and their progeny. They demonstrated that a typical

clone consisted of a radial glial cell, a multipolar daughter cell in the subventricular zone (SVZ), and a neuron migrating along the glial fiber in the intermediate zone (IZ) (Noctor et al., 2001). Using a similar *in vivo* method with GFP-recombinant adenovirus injected into mice, Tamamaki and colleagues demonstrated that four days after infection at E14 or E15, 95% of $GFP^+$ cells were neocortical neurons based on the presence of an axon directed toward the thalamus or corpus callosum (Tamamaki et al., 2001).

These results provided tantalizing evidence that radial glia and the neurogenic precursors of the cerebral cortex are one-in-the-same. In order to definitively address this possibility, Noctor and colleagues employed BrdU, vimentin, phosphorylated vimentin (specific to M phase), RC2, BLBP, and GLAST immunohistochemistry as well as electrophysiological techniques. Their results indicate that nearly all mitotically active VZ cells during neurogenesis possess the morphology and molecular markers of radial glia. In addition, cells demonstrating electrophysiological properties of progenitor cells also exhibit radial glial morphology after intracellular dye filling (Noctor et al., 2002).

Taken together, these data provided sufficient evidence to force a reevaluation of the traditional role of radial glia as static migratory guides. Instead, it became clear that a new perspective was in order, one in which radial glia served the dual function of migratory guides and neurogenic precursors. Such a paradigm shift, of course, brought with it a new set of questions and new lines of investigation.

Molecular marker expression and precursor potential

One such line of investigation included asking whether radial glia are heterogeneous or homogenous with respect to their molecular marker expression and their potential for generating neuronal versus astrocytic progeny. Hartfuss and colleagues

addressed this question utilizing colocalization studies of the radial glial markers RC2, BLBP, GLAST, and nestin during periods of neuro- and gliogenesis in the mouse. Their results indicated a great deal of heterogeneity of marker expression by radial glia in the cortex and ganglionic eminence, with $RC2^+$, $RC2/BLBP^+$, and $RC2/GLAST^+$ cells restricted to the phase of neurogenesis in the cortex, and $GLAST/BLBP^+$ cells appearing at the onset of gliogenesis. Cells in the ganglionic eminence followed a similar pattern, with the notable exception of $RC2/GLAST^+$ precursors persisting into gliogenesis (Hartfuss et al., 2001). Taken together, these data suggest, based on marker expression, that radial glia in the developing CNS are a heterogeneous group of cells.

While the findings of Hartfuss et al. would suggest that separate groups of radial glia may be responsible for neuron and astrocyte production, no direct evidence for this conclusion was presented. Two groups later addressed this question using Cre/loxP *in vivo* fate mapping methods, with the GFAP promoter (Malatesta et al., 2003) or BLBP promoter (Anthony et al., 2004) driving Cre expression in radial glia. The results of Malatesta and colleagues demonstrated pronounced differences between the progeny of radial glia in the cortex versus those in the ganglionic eminence. Cortical radial glia generated nearly all cortical projection neurons but few interneurons (Malatesta et al., 2003). On the contrary, few neurons in the basal ganglia, the derivative of the ganglionic eminence, appeared to originate from ganglionic eminence radial glia. Instead, this population of radial glia generated mostly glial cells (Malatesta et al., 2003).

While these results would suggest that radial glia are also heterogeneous with respect to their potential for generating neurons versus glia, Cre/loxP fate mapping studies conducted by Anthony et al. offer a different conclusion. Utilizing the BLBP

promoter to drive expression of Cre, Anthony et al. demonstrated that radial glia in all brain regions experience a neurogenic period in which they produce most of the neocortical neurons. In addition, their results show that radial glia in the ventral telencephalon complete their neurogenic period earlier than those in the dorsal telecephalon (Anthony et al., 2004). In an effort to reconcile the differences in these data, Anthony and colleagues point to differences in the promoters used to drive Cre expression. While BLBP is already expressed by E12.5 in a rostro-lateral to caudo-medial gradient that parallels the neurogenic gradient, the GFAP promoter does not become active in the ventral telencephalon until E14.5. By this time, according to their results, the ventral telecephalic radial glia have already completed their neurogenic period (Anthony et al., 2004). Whether BLBP promoter activity has begun to drive Cre expression in the ventral telencephalon in the Anthony et al. study prior to the conversion of neuroepithelial cells to radial glia is unclear. Such an occurrence could give the false impression that ventral telencephalic radial glia are neurogenic when these neurons are actually derived from neuroepithelium prior to conversion to radial glia. However, the concept of spatiotemporal heterogeneity of radial glia precursor potential is supported by Li and colleagues after examining coexpression on radial glial cells of BLBP and markers of neuronal precursors (5A5/NCAM) or glial precursors (A2B5) both *in vitro* and *in vivo* (Li et al., 2004). Therefore, it would appear that populations of radial glia across different regions of the telencephalon may be capable of generating neurons, albeit with differing spatiotemporal patterns. Whether individual subsets of radial glia in these locations produce exclusively neurons or glia, or whether all radial glia have the potential to change their daughter cell output *in vivo* over time remains unclear.

A morphological challenge: the radial process during division

Another important question resulting from the paradigm shift toward neurogenic radial glia revolves around the long radial process which distinguishes these cells. Given prior data which suggested that mitotic cells in the developing neural axis rounded up prior to division (Seymour and Berry, 1975), one must account for the radial process during the neurogenic divisions of radial glia, as it would seem teleologically wasteful for these cells to retract their processes, divide, and then re-extend them to provide a substrate for neuronal migration. Data from GFP-recombinant adenovirus infection of E14 mouse neuroepithelium (Tamamaki et al., 2001) and DiI labeling of E14 murine cortical slices (Miyata et al., 2001) suggest that the radial process is retained during division and inherited asymmetrically by one of the daughter cells. Careful examination of radial glia during the G2/M phase transition indicated that the radial process may thin to 0.5μm or less; however, it never retracted or degenerated (Miyata et al., 2001). Moreover, in E14 mouse cortex, the majority of daughter cells receiving the process became neurons and used the process to translocate their somata into the appropriate location in the cortical plate (Miyata et al., 2001; Tamamaki et al., 2001). Simultaneously, the other daughter cell from these divisions re-extended a process with a growth cone-like structure at the leading edge (Tamamaki et al., 2001) at a rate of 20 +/- 8 μm/hr (Miyata et al., 2001), presumably to reestablish radial architecture prior to the next division. In keeping with this concept of radial fiber maintenance during division, several studies of rat cortex after E15 indicate that the radial glial cell maintains its process during mitosis, providing the substrate on which daughter cells can migrate (Noctor et al., 2001; Noctor et al., 2004).

The migratory path of radial glial derived daughter cells

While the above evidence demonstrates that radial fibers are maintained during cell division and inherited asymmetrically, the mode of neuronal migration evident in the E14 mouse data, termed somal translocation, may seem contrary to the established mode of locomotion along radial glial guides (Rakic, 1972; Noctor et al., 2001; Noctor et al., 2004). In fact, somal translocation represents a distinct mode of migration which predominates early in cortical development when the cortex is relatively thin, whereas locomotion becomes more important once the cortical plate expands (Nadarajah et al., 2001; Nadarajah and Parnavelas, 2002; Nadarajah, 2003).

Focusing on the period of neurogenesis during which locomotion predominates, Noctor and colleagues once again employed in utero injection of GFP-recombinant retrovirus into the lateral ventricles of E15/16 rat embryos, and they cultured slices of these brains 24 hours later for time lapse imaging. Interestingly, examination of glial assisted-locomotion of newborn neurons demonstrated that neurons are generated directly by radial glia in the VZ and indirectly via radial glial-derived intermediate progenitors in the SVZ (Noctor et al., 2004). These intermediate progenitor cells (IPCs) have been shown to express some of the same molecular markers as radial glia, such as nestin and phosphorylated vimentin (Miyata et al., 2004; Englund et al., 2005). At the same time, they have been shown to express specific factors that are absent from radial glia, such as Svet1 (Tarabykin et al., 2001), Neurog2 (Miyata et al., 2004), and Tbr2 (Englund et al., 2005), while they fail to express the radial glial transcription factor Pax6 (Englund et al., 2005). Additionally, a progenitor cell type called the short neural precursor (SNP) has been described (Gal et al., 2006), though recent morphological and gene expression

evidence has suggested that SNPs may, in fact, be immature IPCs located adjacent to the apical surface of the ventricular zone, where IPCs are known to originate from radial glial mitoses (Kawaguchi et al., 2008; Kowalczyk et al., 2009). The specific role of intermediate progenitor cells in neocortical neurogenesis is continuing to evolve; however, it is known that these cells contribute not only to the production of upper-layer neurons (Haubensak et al., 2004; Miyata et al., 2004), but also to the production of neurons destined for the preplate and the deeper layers of the cortical plate (Kowalczyk et al., 2009). It has also been proposed that the symmetric neurogenic divisions that these cells undergo may represent an evolutionary strategy for amplifying neuron production in more complex cortices (Martinez-Cerdeno et al., 2006) and for producing area-specific laminar patterns (Pontious et al., 2008).

Regarding the neurons generated directly by radial glia in the work of Noctor and colleagues, the majority underwent four phases of migration en route to the cortical plate (Noctor et al., 2004). During phase one, bipolar neurons ascended from the ventricular surface to the SVZ. During phase two, these neurons arrested in the SVZ and assumed a multipolar morphology, extending and retracting processes while remaining in the SVZ for approximately 24 hours. During phase three, a majority of neurons extended a leading process and migrated retrogradely to the ventricle. Upon contacting the ventricle, they reversed polarity and ascended to the cortical plate during phase four. Alternatively, a minority of neurons bypassed phase three in favor of phase four migration to the cortical plate (Noctor et al., 2004). While the purpose of such phasic migration is yet unknown, it is intriguing to speculate, as Noctor and colleagues do, that a sojourn in the SVZ with subsequent retrograde migration may be important for receiving positional

cues or for coordinating migration with ganglionic eminence-derived interneurons destined for the same layer. Additionally, a defect that arrests a neuron in phase two of migration may have clinical significance as a mechanistic explanation for cortical malformation disorders such as subcortical band heterotopia (Kriegstein and Noctor, 2004; Noctor et al., 2004).

**Transcription factor cascades in glutamatergic neurogenesis**

Since the description of the direct and indirect modes of neurogenesis discussed above, significant effort has gone into delineating the genetic programs and transcription factor cascades responsible for the transition from neurogenic radial glial cell to post-mitotic glutamatergic projection neuron. Resulting from this effort has been the identification of a relatively conserved transcription factor cascade that is spatiotemporally correlated with maturation of a radial glial cell into a glutamatergic neuron in both the dorsal neocortex and the cerebellum. The cascade follows a progression from Pax6 → Neurog2 → Tbr2 → NeuroD → Tbr1, in which the transcription factors are expressed by cells in the VZ, VZ/SVZ, SVZ, IZ, and CP, respectively (Figure 1-3) (Hevner, 2006; Hevner et al., 2006; Hodge et al., 2008).

The first member of this cascade, Pax6, is a paired-homeodomain containing transcription factor with critical roles in the regulation of diverse developmental processes, including neocortical area patterning, upper-layer identity, cell cycle kinetics, mitotic cleavage plane, neuronal fate, forebrain axon growth and guidance, cell adhesion,

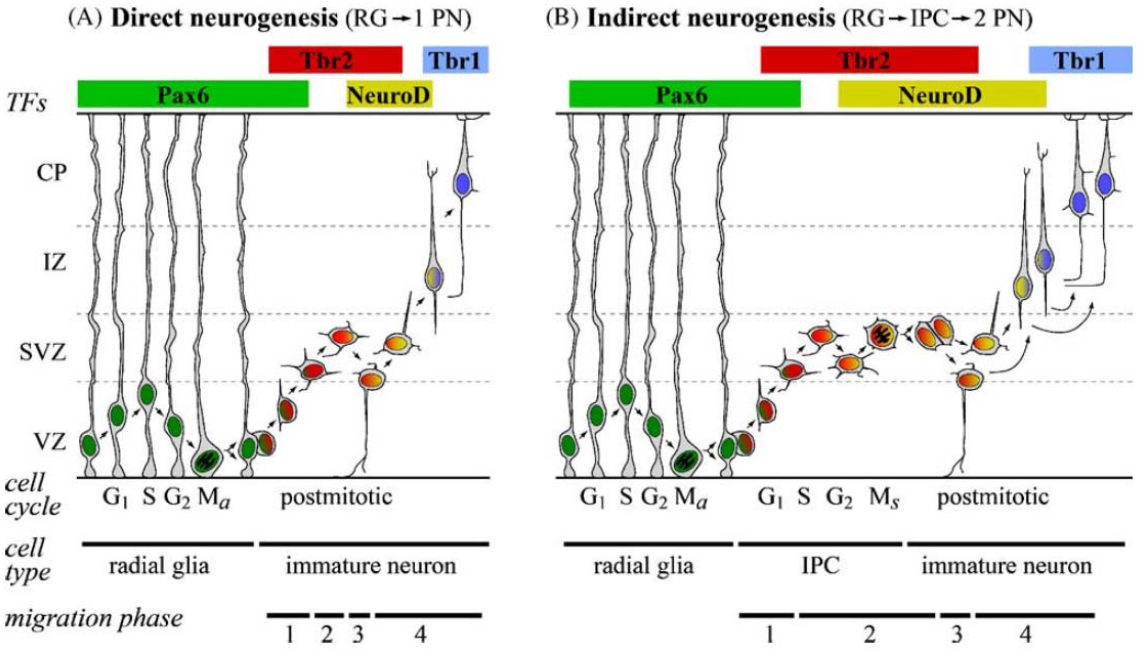



**Figure 1-3. Trascription factor cascades during direct and indirect neurogenesis.** Production of glutamatergic neurons either directly from radial glia or indirectly from intermediate progenitors is accompanied by a sequence of transcription factor expression. These transcription factors are expressed at specific stages of evolution from radial glia to post-mitotic neurons and control genetic programs that are important for the acquisition of cell type specific features. (from Hevner et al., 2006)

cell migration, and glutamatergic identity (Caric et al., 1997; Bishop et al., 2000; Tarabykin et al., 2001; Estivill-Torrus et al., 2002; Heins et al., 2002; Hevner et al., 2002; Jones et al., 2002; Tyas et al., 2003; Schuurmans et al., 2004; Kroll and O'Leary, 2005; Quinn et al., 2007). Within the VZ, Pax6 is expressed chiefly by radial glial cells, but also by daughter cells of radial glial divisions which are migrating en route to the SVZ as they are maturing into $Tbr2^{+}$ IPCs (Englund et al., 2005; Hevner et al., 2006). Neurog2 is a proneural transcription factor that is regulated by Pax6 (Scardigli et al., 2003) and that has been shown to convert ventricular surface-dividing cells into non-surface dividing cells (Miyata et al., 2004) which subsequently express Tbr2 (Englund et al., 2005) as they migrate into the SVZ. Recently, Neurog2 has also been identified as a transcriptional inducer of Rnd2, a small GTP-binding protein that is critical for radial migration of newborn neurons, suggesting that proneural factors responsible for initiating neuronal differentiation programs may also coordinate migration in the differentiating cells (Heng et al., 2008).

Upon its arrival in the SVZ, an IPC finishes downregulating Pax6 and upregulating the T-box transcription factor Tbr2, thus marking its maturation. Little is known about the transcriptional program induced by Tbr2; however, recent gene array evidence in Pax6 deficient mice has demonstrated a strong downregulation of Tbr2, which supports the notion that Pax6 expression is required for induction of Tbr2 during development (Holm et al., 2007). In addition, loss of Tbr2 expression leads to a decrease in cortical surface area, thickness, and neuron number in all cortical layers, while misexpression of Tbr2 in the VZ induces IPC identity in radial glial cells (Arnold et al., 2008; Sessa et al., 2008).

NeuroD is a basic helix-loop-helix (bHLH) transcription factor of particular importance in the pancreas and brain where it functions as a differentiation factor. In the developing brain, NeuroD is critical for the survival and differentiation of neurons in the dentate gyrus and the cerebellum (Chae et al., 2004). In support of the concept of NeuroD as a downstream target of Tbr2 in the glutamatergic neurogenic transcription factor cascade, the two factors have been shown to be coexpressed in some IPCs in the SVZ (Hevner et al., 2006). Also, Pax6 deficient mice downregulate NeuroD and Neurog2, a transcription factor that can increase the number of non-surface dividing progenitors as described above (Miyata et al., 2004) and that is expressed in cells that later express NeuroD (Chae et al., 2004). Tbr1, the final transcription factor in the glutamatergic neurogenic cascade, is expressed in virtually all pyramidal projection neurons (Hevner et al., 2006) and may regulate some overarching aspects of the glutamatergic phenotype, in addition to having important roles in specifying deep layer-identity, cell migration, and axon pathfinding (Hevner et al., 2001; Hevner et al., 2002; Fink et al., 2006).

**Coordination of migration, cell cycle exit, and differentiation in neocortical development**

During development of the rodent neocortex, radial glia and intermediate progenitor cells are the principal source of glutamatergic projection neurons. After their birth in the proliferative compartments, daughter cells migrate toward the pial surface, exit the cell cycle, and initiate differentiation. While the molecular mechanisms coordinating these processes are still being elucidated, it is clear that multifunctional

proteins play an important role. Indeed, the proneural factor Neurog2 is responsible for the expression of genes important for neuronal differentiation; however, it also initiates and maintains migration away from the ventricle (Miyata et al., 2004; Britz et al., 2006; Heng et al., 2008). Moreover, Neurog2 has been shown to alter the expression of other important regulators of migration, such as p35, DCX, and RhoA (Ge et al., 2006). Likewise, $p27^{kip1}$, which is important for $G_1$ arrest and cell cycle exit, has been shown to stabilize Neurog2 and inhibit RhoA signaling (Nguyen et al., 2006). In addition, $p27^{kip1}$ can be phosphorylated and stabilized by CDK5, an effector of the reelin pathway that signals to migrating neurons, and this stabilized form promotes the actin organizing function of cofilin, thus enhancing migration (Kawauchi et al., 2006). Additional studies have demonstrated that CDK5 is necessary for neuronal differentiation and cell cycle inhibition, that the Cdk inhibitor $p57^{kip2}$ also has pro-migration effects, and that the cell cycle regulating retinoblastoma (Rb) protein serves another role as a controller of migration (Ferguson et al., 2002; Cicero and Herrup, 2005; Ferguson et al., 2005; Itoh et al., 2007). Despite this intricate coordination, these processes can be uncoupled, suggesting that inappropriately timed expression of critical molecules, whether via induction or failed downregulation of gene expression, can cause cells to migrate out of proliferative compartments without first exiting the cell cycle (Zindy et al., 1999; Cicero and Herrup, 2005; Lobjois et al., 2008).

**Cortical malformation: disordered cellular genesis and the tish rat**

While it is clear that lissencephaly/SBH resulting from mutations in DCX or Lis1 lends itself to classification as a neuronal migration disorder, it is equally apparent that

alternative interpretations for the pathogenesis of this disorder may exist, as the genetic underpinnings of 10-20% of lissencephaly and SBH cases are yet unknown (Delatycki and Leventer, 2009). Although the remaining cases of lissencephaly/SBH may result from neuronal migration defects due to genes which are yet uncharacterized, some authors have previously suggested a role for disordered cellular proliferation or apoptosis in lissencephaly/SBH (Rorke, 1994; Eksioglu et al., 1996; Lee et al., 1998a). These interpretations leave open the possibility that lissencephaly/SBH can be modeled by disrupting processes other than migration, and, more importantly, that the pathogenesis of the unexplained human cases may in part be elucidated by considering the problem from a non-migration perspective. The studies described in this thesis evaluate neocortical development in the tish rat, a novel neurological mutant exhibiting autosomal recessive SBH as well as behavioral and electroencephalographic seizures. Evidence from the development of the tish neocortex suggests that the band heterotopia in this model is formed as a result of mislocalized cellular proliferation in the intermediate zone and cortical plate, suggesting an alternative pathogenetic mechanism for establishing a lissencephalic defect.

A novel model of subcortical band heterotopia

The tish rat (telencephalic internal structural heterotopia) is a neurological mutant displaying bilateral heterotopia in the white matter of the frontal and parietal, but not temporal, neocortex. Accompanying the heterotopia is a variable degree of ventriculomegaly. In these animals, the gray matter of the neocortex exists as two cortices, one normotopic which exhibits typical features including laminar organization, and one heterotopic which contains similar cellular elements but lacks a clear laminar

organization (Figure 1-4). Instead, the heterotopic cortex is organized in a rim-to-core fashion whereby the earliest born neurons take up residence around the rim of the heterotopia and the latter born neurons apparently migrate past them to the core of the structure (Lee et al., 1997). Measurements of cortical volume in adult tish animals (normotopic + heterotopic cortex) have indicated no difference in overall neocortical volume compared with controls; rather, the heterotopia appears to occur at the expense of the normotopic cortex, rendering it thinner in areas where the heterotopia is present (Lee et al., 1999). Interestingly, despite the layering defects of the heterotopia and the tendency for pyramidal neurons in this region to orient their dendrites in a non-uniform manner, both normotopic and heterotopic pyramidal neurons are capable of establishing appropriate subcortical connections in sensory and motor cortex, indicating intact axon pathfinding capabilities in the tish mutant (Lee et al., 1997; Schottler et al., 1998; Schottler et al., 2001).

Similar to human patients with SBH, the tish rat exhibits behavioral and electroencephalographic seizures, as well as deficits in learning and memory. Some homozygous tish animals experience partial seizures with secondary generalization that affect predominantly neocortical and limbic structures, rather than subcortical structures (Lee et al., 1997; Chen et al., 2000). On the other hand, heterozygous animals, which do not possess a heterotopia, do not experience seizures, indicating that some feature of the heterotopia itself or of the normotopic cortex dorsal to the heterotopia is likely responsible for seizure susceptibility. Indeed, *in vitro* examination of neuronal spiking activity in tish cortex in the presence of proconvulsant compounds indicated that aberrant discharge activity is initiated in the normotopic cortex before subsequently recruiting the

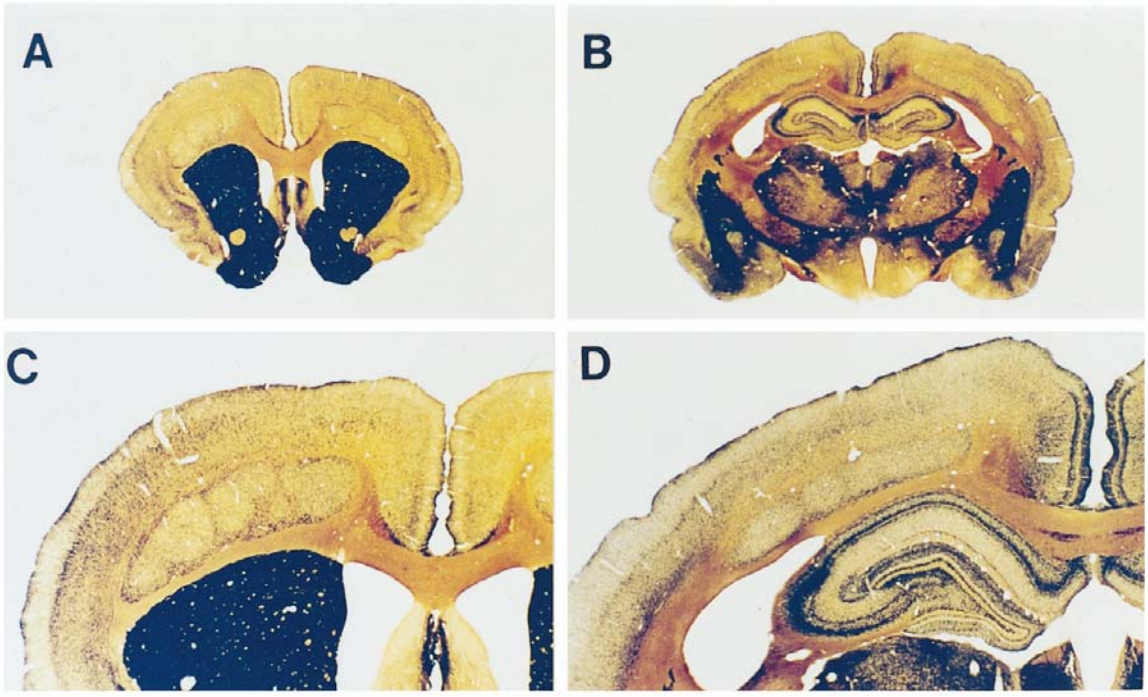



**Figure 1-4. Histological appearance of the adult tish neocortex.** Low power (**A-B**) and higher power (**C-D**) images of acetylcholinesterase stained tish brains demonstrate the subcortical band heterotopia contained within the white matter of the neocortex. Tish heterotopia exist in frontal and parietal regions, but spare the occipital and temporal cortices (from Lee et al., 1997).

heterotopic cortex (Chen et al., 2000). While the cellular and molecular substrates of tish epileptogenesis are still under investigation, recent evidence indicates that key elements of GABAergic transmission are disturbed before seizure onset in the tish neocortex, including a reduction in the number of parvalbumin- positive interneurons in the normotopic cortex (Trotter et al., 2006). These findings represent a rational substrate for tish epileptogenesis.

Regarding the learning and memory assessment of tish animals, preliminary studies have utilized the Morris Water Maze and an 8-arm radial maze to examine spatial learning. Results from the Morris Water Maze indicated no difference between homozygous tish animals and controls. However, results from a more complex task on the 8-arm maze which requires the animal to employ a win-shift strategy indicated that both homozygous tish and heterozygous animals exhibited a learning deficit (unpublished data). Taken together, these results suggest two important conclusions. First, the learning and memory deficit exhibited by tish animals may be subtle, requiring a more complex task to resolve. Second, heterozygous animals, which do not possess a heterotopia, nevertheless display deficits on more complex learning tasks, suggesting a specific phenotype in heterozygotes that depends upon more subtle cellular defects due to haploinsufficiency for the tish gene.

Basic features of tish cortical development

Another important line of investigation centers on the origin of the subcortical band heterotopia in the tish mutant. While the responsible gene has not yet been identified, the inheritance pattern is consistent with a single gene defect (Lee et al., 1997). Therefore, investigation into tish cortical development has focused on characterizing the

tish phenotype. Improper formation of the preplate, marginal zone, or subplate during development could affect the integrity of the tish cortex. Investigation of these structures using calretinin reactivity at E15, E18, and E20 demonstrated that, comparable to controls, only one preplate forms in a normotopic position in tish animals which subsequently splits into a single marginal zone and subplate during expansion of the normotopic cortical plate. In contrast, the heterotopic cortical plate within the intermediate zone forms without a dedicated preplate (Lee et al., 1998a).

While preplate, subplate, and marginal zone formation appear normal in the tish mutant, it is conceivable that defects in Cajal-Retzius (CR) cell distribution or their secreted product, reelin, may lead to errors in cortical development (see Tissir and Goffinet, 2003 for a review of reelin function in development). Investigation of CR cells using the CR-50 antibody that recognizes reelin revealed that reelin distribution and CR cell localization was similar between tish mutants and control animals (Lee et al., 1998a). Taken together with the other structural differences between the tish and reeler mutants, particularly the lack of an inverted cortex in tish, these results suggest that the reelin pathway is intact in tish animals.

While the tish phenotype is most similar to SBH of doublecortin origin, the unique feature in tish cortical genesis is a region of mislocalized cellular proliferation during development. Two-hour bromodeoxyuridine (BrdU) pulse labeling experiments at E15 demonstrated S-phase cells in a discreet band within the VZ/SVZ of control animals, as compared with tish cortex in which S-phase cells are dispersed throughout the width of the developing dorsal cortex. Similar experiments at E18 showed BrdU positive cells in the VZ of control neocortex, whereas positive cells in tish neocortex were found in the

VZ as well as in a heterotopic location in the superficial intermediate zone dorsal to the developing heterotopia. These results were corroborated by PCNA immunohistochemistry which also labels mitotically competent cells (Lee et al., 1998a). Taken together, these results reveal abnormal cellular proliferation in tish neocortex and suggest that heterotopic cellular genesis might play a role in creating the SBH that characterizes the tish malformation.

Radial glial contribution to the tish phenotype

Given the recent characterization of radial glia and intermediate progenitor cells as neurogenic (Noctor et al., 2001; Noctor et al., 2002; Noctor et al., 2004) and the finding of heterotopic cellular genesis in the tish cortex (Lee et al., 1998a), my working hypothesis of tish neurogenesis is that heterotopic mitotic cells in the IZ/CP are either radial glia or intermediate progenitor cells and that one or both of these cell types lead to the formation of the SBH. It is possible that heterotopic proliferative cells might produce neurons directly and that these neurons might migrate ventrally into the SBH rather than dorsally into the CP. Alternatively, heterotopic proliferative cells might exacerbate or induce aberrant neuronal migration from the VZ/SVZ by disrupting normal migratory cues. Regardless of which of these scenarios underlies the structural malformation in the tish neocortex, this animal model represents an alternative mechanism of SBH formation, which, upon further characterization and identification of the responsible gene(s), will enhance our understanding of normal cortical development and may offer an explanation of the pathogenesis of human lissencephaly cases that are thus far unexplained.

**Experimental Design**

The central hypothesis of the work presented in this thesis is that cells of radial glial and intermediate progenitor cell lineages are present heterotopically in the developing tish neocortex and contribute to the formation of the subcortical band heterotopia in the tish brain. Two chapters follow that present experiments designed to test this hypothesis.

**Chapter 2: The tish mutation disrupts progenitor positioning and cell cycle regulation during neocortical development**

The heterotopic, BrdU-incorporating cells identified previously in the tish$^{-/-}$ neocortex could have several identities. It is possible that the tish mutation could result in the mislocalization of progenitor cells of the neuronal or glial lineage in the developing neocortex. Alternatively, the tish mutation could cause migrating, differentiating neurons to retain characteristics of a previous progenitor cell identity, such as transcription factor markers or cell cycle activity (Hevner, 2006). Previous studies have indicated that it is possible to uncouple the processes of cell cycle arrest and differentiation in migrating neurons (Zindy et al., 1999; Cicero and Herrup, 2005; Lobjois et al., 2008). Thus, the current study examined the expression of cell-type specific markers in order to characterize the heterotopic BrdU$^{+}$ cells in tish$^{-/-}$ neocortex. In addition, an iododeoxyuridine/bromodeoxyuridine assay was used to establish the cell cycle parameters of heterotopic, BrdU-incorporating cells, in order to determine whether the dynamics of progenitor lineage cells are disturbed when such cells are positioned inappropriately.

## Chapter 3: Cellular mechanisms underlying progenitor mislocalization in the tish neocortex

During normal neocortical development, daughter cells born in the VZ/SVZ must exit the cell cycle, begin migration toward the cortical plate, and initiate differentiation. It is clear that multifunctional proteins such as Neurog2, $p27^{kip1}$, $p57^{kip2}$, and Rb play an important role in the coordination of cell cycle exit, migration, and differentiation in these cells (Ferguson et al., 2002; Miyata et al., 2004; Britz et al., 2006; Kawauchi et al., 2006; Nguyen et al., 2006; Itoh et al., 2007; Heng et al., 2008). It is also known that these processes can be uncoupled (Zindy et al., 1999; Cicero and Herrup, 2005; Lobjois et al., 2008) and that failure of cell-cell junctions between parental radial glia can lead to heterotopic mitoses (Cappello et al., 2006; Imai et al., 2006; Lien et al., 2006). Thus, it is possible that heterotopic $BrdU^{+}$ cells in the $tish^{-/-}$ neocortex may become mislocalized via a failure of cell-cell junctions within the VZ or a failure of VZ/SVZ-born daughter cells to efficiently initiate differentiation while also exiting the cell cycle as they migrate toward the cortical plate. The current study examined the cellular mechanisms underlying progenitor cell mislocalization in the tish neocortex using immunohistochemistry to examine cell-cell junctions in the VZ and *in utero* electroporation of a pCAGGS-GFP construct to follow VZ-born daughter cells as they migrate toward the cortical plate.

# Chapter 2

## The tish mutation disrupts progenitor positioning and cell cycle regulation during neocortical development

## Abstract

While theories focusing on defective neuronal migration have yielded causative genes and pathogenic mechanisms for certain human cases of SBH, others still exist for which no causative gene can be identified, leaving open the possibility for alternative mechanisms of SBH formation. My studies used a seizure-prone genetic animal model of SBH, the tish rat, which differs from other animal models of this disorder due to the presence of an abnormally located (heterotopic) band of proliferating cells in the intermediate zone and cortical plate. In order to characterize these heterotopic, mitotically competent cells, this study employed immunohistochemical methods to detect markers of progenitor and neuronal lineages, as well as an iododeoxyuridine/ bromodeoxyuridine (IdU/BrdU) assay to characterize cell cycle kinetics.

The results indicated that heterotopic proliferating cells in tish$^{-/-}$ and tish$^{+/-}$ neocortex incorporated BrdU and expressed the M-phase marker phosphorylated vimentin as well as cell type specific markers of radial glia and intermediate progenitor cells. These cells did not express β-III tubulin, a marker of immature neurons, indicating that they had not yet begun to differentiate. Furthermore, cell counts of Pax6$^{+}$ and Tbr2$^{+}$ cells in wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices demonstrated that cell density was increased in tish$^{-/-}$ neocortex from E17 to E20. Kinetic analyses showed that heterotopic progenitors in the tish$^{-/-}$ neocortex possessed a shortened cell cycle length largely due to a decreased time spent in $G_2$+M+$G_1$ phases. In contrast, progenitors in the tish$^{+/-}$ neocortex possessed either a shortened or elongated cell cycle length depending on embryonic age due largely to changes in $G_2$+M+$G_1$ phases. These data demonstrate that the positioning

and cell cycle behavior of progenitor cells are fundamentally altered beginning in the early and middle stages of cortical plate development in the tish neocortex.

## Introduction

Normal development of the mammalian neocortex is a complex process of precisely timed molecular and cellular events, the details of which continue to be elucidated and refined. Considering this complexity, it is hardly surprising that errors occur, resulting in an overall incidence of cortical malformation in humans of around 1%, with an incidence of approximately 14% in patients with epilepsy and approximately 40% in patients with intractable epilepsy (Hardiman et al., 1988; Farrell et al., 1992; Meencke and Veith, 1992). One of the major categories of cortical malformation, classical lissencephaly, results in a neocortex that exhibits agyria, pachygyria, or subcortical band heterotopia pathologically, and some degree of mental retardation and epilepsy clinically (Dobyns et al., 1993; Dobyns et al., 1996). Research into the genetic causes of this disease spectrum has identified mutations in genes such as doublecortin (DCX), lissencephaly-1 (Lis1), aristaless-related (Arx), α-tubulin (TUBA3), reelin (RELN), very-low density lipoprotein receptor (VLDLR), and ApoE receptor 2 (ApoER2), which are critical for effective migration of newborn neurons from their birthplace in the ventricular and subventricular zones to their destined layer within the cortical plate (Reiner et al., 1993; Hirotsune et al., 1995; des Portes et al., 1998b; Trommsdorff et al., 1999; Kitamura et al., 2002; Keays et al., 2007). These findings have reinforced the concept that most classical lissencephaly-spectrum malformations of the neocortex result from a primary defect in neuronal migration. Despite these advances in our understanding, the causative gene(s) and cellular mechanisms underlying some human cases of this disorder remain elusive (Delatycki and Leventer, 2009).

The present study utilizes a novel model of subcortical band heterotopia (SBH), the tish rat, to investigate the cellular mechanisms underlying SBH formation during embryonic development. The tish rat is a spontaneously occurring genetic model of SBH in which the malformation is inherited in an autosomal recessive manner (Lee et al., 1997). A unique feature of the developing tish$^{-/-}$ neocortex is the presence of an abnormally located (heterotopic) band of proliferating cells in the intermediate zone and cortical plate in addition to the normally-positioned (normotopic) band of cells in the ventricular and subventricular zones (Lee et al., 1998a). The present studies utilize immunohistochemistry to characterize the identities and cell cycle kinetics of these cells. A broader goal of this research is to elucidate causative mechanisms for SBH formation that may be of benefit in understanding the aforementioned human cases whose causative mechanisms remain elusive.

Overall, the results demonstrate that the tish mutation disrupts the position and cell cycle kinetics of progenitors in the developing neocortex. Specifically, Pax6$^{+}$ radial glia and Tbr2$^{+}$ intermediate progenitors are found in a heterotopic location in the intermediate zone and cortical plate of tish$^{-/-}$ neocortex and, to a lesser extent, in a similar location in tish$^{+/-}$ neocortex. These heterotopic Pax6$^{+}$ and Tbr2$^{+}$ cells in tish$^{-/-}$ and tish$^{+/-}$ neocortices incorporate BrdU and express phosphorylated vimentin, while failing to express β-III tubulin, confirming that heterotopic proliferating cells are cycling progenitors and not immature neurons. Furthermore, Pax6$^{+}$ and Tbr2$^{+}$ cell density is increased in tish$^{-/-}$ neocortex compared with wildtype and tish$^{+/-}$ cortex, and the cell cycle

kinetics of tish$^{+/-}$ and heterotopic tish$^{-/-}$ progenitors are dysregulated, providing a logical explanation for the increase in cell density.

## Materials and Methods

### Animals and breeding

Animals were housed at 22°C on a standard 12h:12h light–dark schedule with free access to food and water. Animals were handled according to NIH guidelines and a protocol approved by the University of Virginia Animal Care and Use Committee. The tish phenotype is expressed on a Sprague-Dawley background, and the heterotopia are inherited in an autosomal recessive manner, requiring two mutated alleles in order to display the SBH phenotype. Therefore, timed pregnant litters of $tish^{-/-}$ pups were generated by mating a $tish^{-/-}$ male with a $tish^{-/-}$ female. Wildtype Sprague-Dawley control litters were generated by mating a wildtype male to a wildtype female. $Tish^{+/-}$ litters were generated by mating a $tish^{-/-}$ male to a wildtype female. In all cases, the morning of vaginal plug discovery was designated as embryonic day E0.5.

### Bromodeoxyuridine administration

For those animals used in the immunohistochemical characterization of tish progenitor cells, bromodeoxyuridine (BrdU) was administered as previously described (Lee et al., 1998a). Briefly, pregnant dams were given an intraperitoneal injection of BrdU (50 mg/kg, Sigma) and then euthanized 2h later under deep anesthesia. The brains of the embryos were then removed and prepared for sectioning. This administration protocol was employed to label only those progenitor cells within S phase or about to exit S phase at the time of administration, since a two-hour survival is insufficient time for these cells to complete mitosis and pass BrdU on to their progeny (Takahashi et al., 1995).

**Tissue processing and Immunohistochemistry**

Dams with timed pregnancies were anesthetized with isoflurane and decapitated, and embryos were removed between embryonic day E15 and E20. Embryonic brains were rapidly dissected in 0.1M PBS and fixed for 1h in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose until sinking. Cryostat sections were cut at 10 or 20μm and mounted on Superfrost Plus slides (Fisher Scientific).

Slide mounted sections were boiled briefly in 10mM sodium citrate pH 6 to enhance antigen recognition, followed by incubation in 2N HCl for 30 min to expose BrdU or IdU. Sections were then blocked in 5% normal goat serum (Vector Laboratories, Burlingame CA) with 0.3% Triton X100 in 0.1M PBS for 2h before overnight incubation at 4˚C with primary antibody diluted in blocking serum. The following primary antibodies were used: anti-Pax6 (rabbit, 1:300, Covance), anti-Tbr2 (rabbit, 1:300, Millipore), anti-phosphorylated vimentin 4A4 clone (mouse, 1:500, Assay Designs), anti-BrdU (mouse, 1:10, BD Biosciences), anti-BrdU (rat, 1:40, Abcam), anti-βIII tubulin (mouse, 1:100, Sigma-Aldritch), anti-cleaved caspase 3 (rabbit, 1:200, Cell Signaling Technology). Incubation with secondary antibody diluted in blocking serum was performed for 1h at room temperature. For cell counting of $Pax6^+$ and $Tbr2^+$ cells, a goat anti-rabbit biotinylated antibody (1:250, Vector Laboratories) was used, followed by detection via the ABC method (Vector Laboratories, Burlingame CA) with 3,3'-diaminobenzidine (DAB, Sigma-Aldritch) as chromagen. For immunofluorescence, the following secondary antibodies were used: goat anti-rabbit Alexa 633, goat anti-rabbit 488, goat anti-mouse Alexa 488, goat anti-mouse 594, or goat anti-rat Alexa 594 (all at 1:250, Invitrogen), followed by incubation with 4',6-diamidino-2-phenylindole (DAPI)

for 5 min. Finally, in the case of the DAB developed sections, slides were dehydrated in graded ethanols, cleared in Xylenes, and coverslipped with Permount (Fisher Scientific), or in the case of fluorescent sections, slides were air dried, coverslipped with ProLong Gold anti-fade reagent (Invitrogen), and stored at -20˚C. Light microscopic images of DAB labeled sections were captured by a Zeiss Axiocam camera mounted on a Zeiss Axioplan2 microscope. Fluorescent images were captured on a Zeiss LSM510 confocal microscope.

**Cell counting and cell cycle assay**

Quantification of $Pax6^{+}$ and $Tbr2^{+}$ cell density was performed on sections of wildtype, $tish^{+/-}$, and $tish^{-/-}$ embryos from E15 through E20. $Pax6^{+}$ and $Tbr2^{+}$ nuclei were counted within a defined area that spanned the entire cortical width from ventricular to pial surface in two non-consecutive sections from five embryos for each genotype and developmental day. One rostral and one caudal section were utilized for each embryo, and measurements were taken in the medial and lateral dimensions of each section. Rostral sections were chosen based on the presence of the medial and lateral ganglionic eminences, while caudal sections were chosen based on the presence of the developing hippocampus, thalamus, and hypothalamus. Lateral cortex was considered to be the width of a 10x microscopic field beginning at the pallial-subpallial border and continuing medially. Medial cortex was considered to be a 10x microscopic field of cortex bordering the longitudinal fissure. Data are expressed as the number of cells/$mm^2$ and p-values are the result of a Kruskal-Wallis One-way ANOVA with a Tukey's multiple comparison test or a One-way ANOVA with a Holm-Sidak multiple comparisons test

comparing wildtype, tish$^{+/-}$, and tish$^{-/-}$ animals at each embryonic age. All statistical analyses were performed using SigmaStat software (SYSTAT Software, Inc.).

In order to assess cell cycle parameters within Pax6$^{+}$ and Tbr2$^{+}$ progenitor populations, an IdU/BrdU labeling method was used as previously described (Quinn et al., 2007). Briefly, at E17 or E20, timed-pregnant dams were injected i.p. with IdU followed by BrdU (both at 50mg/kg) 1.5h later. Dams were sacrificed 2h after the first injection and embryos were removed, fixed, and sectioned as detailed above. Triple immunofluorescence was performed as indicated utilizing either an anti-Pax6 or anti-Tbr2 antibody to label the total population of progenitor cells, in conjunction with an anti-BrdU antibody which recognizes both IdU and BrdU (mouse, BD Biosciences) and an anti-BrdU antibody which only recognizes BrdU (rat, Abcam). Confocal images were imported into ImageJ (National Institutes of Health), and the proportions of IdU/BrdU labeled cells in the proliferative zones of the dorsal neocortex were counted in two non-consecutive sections from 3-5 embryos for each genotype and developmental day. Cell cycle lengths were calculated as follows: cells that left S-phase during the 1.5 hr interval between the two injections (IdU$^{+}$ only) were designated the leaving fraction ($L_{cells}$). Cells that remained in S phase for the entire 2 hr duration (IdU and BrdU$^{+}$) or that entered S phase after the second injection (BrdU$^{+}$ only) were designated the S-phase cells ($S_{cells}$). Cell cycle parameters were then calculated as follows: S phase length ($T_s$) $\rightarrow$ $T_s/1.5 = S_{cells}/L_{cells}$; cell cycle length ($T_c$) $\rightarrow$ $T_c/T_s = P_{cells}/S_{cells}$, where $P_{cells}$ represents the total number of proliferating cells as indicated by Pax6 or Tbr2 immunoreactivity; percentage of time spent in S phase $\rightarrow$ $T_s/T_c$; length of $G_2$+M+$G_1$ $\rightarrow$ $T_c$-$T_s$. Data are expressed as mean ± SEM and p-values are the result of either a One-way ANOVA with the Holm-

Sidak multiple comparisons test or a Kruskall-Wallis one-way ANOVA with Dunn's multiple comparisons testing.

## Results

### Cortical progenitor cells are incorrectly positioned in the tish$^{-/-}$ neocortex

Given recent evidence that radial glial cells and intermediate progenitor cells (IPCs) are neurogenic (Noctor et al., 2001; Noctor et al., 2002; Noctor et al., 2004), we sought to characterize the abnormally-positioned, mitotically-competent cells that have been previously identified in the intermediate zone (IZ) and normally-positioned cortical plate (CP) of the developing tish$^{-/-}$ neocortex (Lee et al., 1998a). Toward this end, we utilized immunohistochemistry to visualize Pax6$^{+}$ radial glia and Tbr2$^{+}$ intermediate progenitor cells (IPCs) (Englund et al., 2005). Examination of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices at time points corresponding to early, mid, and late cortical plate neurogenesis demonstrated that the heterotopic proliferative zone of the tish$^{-/-}$ cortex contains both Pax6$^{+}$ and Tbr2$^{+}$ cells (Fig. 2-1 and 2-2). Early in cortical plate neurogenesis at E15, small groups of inappropriately located Pax6$^{+}$ cells were found in the tish$^{-/-}$ preplate region just beneath the pial surface (Fig. 2-1C arrows, compare with Fig. 2-1A and 2-1B). These misplaced cells were present in addition to the normally-positioned Pax6$^{+}$ cells in the ventricular zone (VZ) but were not observed in the corresponding preplate region in either wildtype or tish$^{+/-}$ cortex. As development proceeded, these small groups of heterotopic Pax6$^{+}$ cells coalesced into a diffuse band in the IZ and CP by E17 (Fig. 2-1F arrows, compare with Fig. 2-1D and 2-1E). This band of mislocalized cells persisted through E20 (Fig. 2-1I arrows, compare with Fig. 2-1G and 1H). Interestingly, at E17 in the tish$^{+/-}$ neocortex, a few small clusters of Pax6$^{+}$ cells

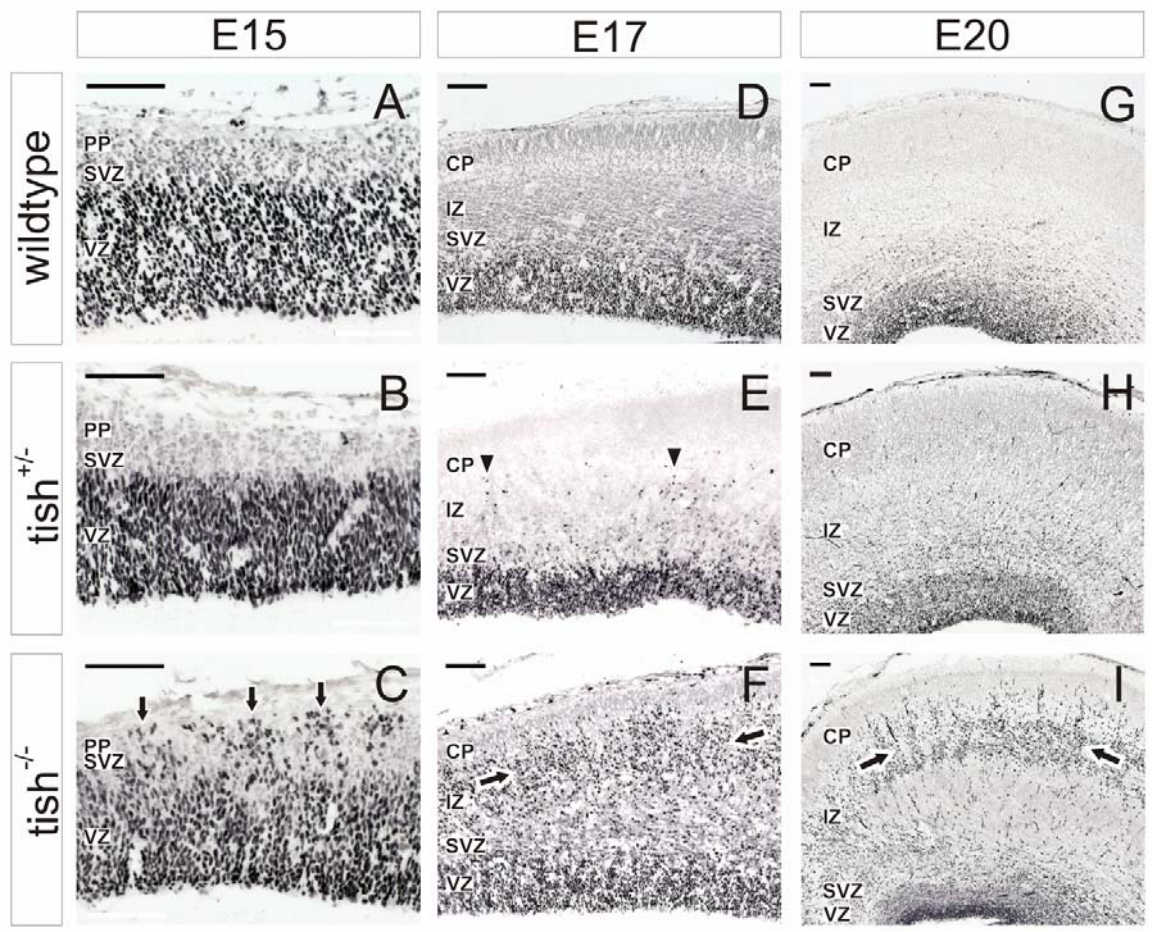



**Figure 2-1. Pax6$^{+}$ cells are incorrectly positioned in the tish$^{-/-}$ neocortex.** Light microscopic images were taken of coronal sections of embryonic neocortex during timepoints corresponding to early, middle, and late cortical plate neurogenesis. **A-C**, Early during cortical plate neurogenesis (E15), clusters of inappropriately positioned Pax6$^{+}$ cells can be found in a superficial position in the vicinity of the preplate (PP) of tish$^{-/-}$ neocortex (arrows). These cells are normally restricted to the ventricular zone (VZ) in wildtype and tish$^{+/-}$ animals. **D-F**, During the middle stage of cortical plate neurogenesis (E17), a diffuse band of inappropriately positioned Pax6$^{+}$ cells is observed in the intermediate zone (IZ) and cortical plate (CP) of tish$^{-/-}$ neocortex (arrows). These cells are normally restricted to the VZ in wildtype and tish$^{+/-}$ neocortex. **G-I**, Late in cortical plate neurogenesis (E20), the diffuse band of inappropriately positioned Pax6$^{+}$ cells persists in the IZ/CP of tish$^{-/-}$ neocortex (arrows). These cells are normally restricted to the VZ in wildtype and tish$^{+/-}$ neocortex at this age. Scale bars = 100μm.

were present in the tish$^{+/-}$ IZ, suggesting a partial gene dosage effect for the tish gene in the positioning of Pax6$^{+}$ progenitor cells during development (Fig. 2-1E, arrowheads). Despite this partial positioning defect, in tish$^{+/-}$animals, subcortical band heterotopia are observed only in tish$^{-/-}$ animals and not in tish$^{+/-}$ animals, suggesting that a greater number of mislocalized cells are required before the adult structural malformation can be established.

Tbr2$^{+}$ cells also exhibit a positioning defect in the developing tish neocortex. Beginning on E15, scattered Tbr2$^{+}$ cells were observed in the tish$^{-/-}$ preplate region separate from the primary population of Tbr2$^{+}$ cells, which was located in the developing subventricular zone (SVZ) (Fig. 2-2C arrows, compare with Fig. 2-2A and 2-2B). By E17, a diffuse band of mislocalized Tbr2$^{+}$ cells was located in the IZ and CP (Fig. 2-2F, compare with Fig 2-2D and 2-2E) and persisted in this location through E20 (Fig. 2-2I, compare with Fig. 2-2G and 2-2H). Interestingly, at E17 in tish$^{+/-}$ neocortex, a few small clusters of Tbr2$^{+}$ cells were observed in the tish$^{+/-}$ IZ, consistent with a gene dosage effect for the tish gene in the positioning of Tbr2$^{+}$ progenitor cells during development (Fig. 2-2E, arrowheads). These results suggest that the primary mechanistic defect in progenitor cell positioning in the tish$^{-/-}$ neocortex likely resides in the radial glial population, since Pax6$^{+}$ cells were more severely affected than Tbr2$^{+}$ cells at E15. The increasingly mislocalized Tbr2$^{+}$ cells at later stages are likely a direct consequence of these cells being generated by previously mislocalized radial glia; however, additional seeding of the heterotopic proliferative zone by the normally-positioned VZ/SVZ

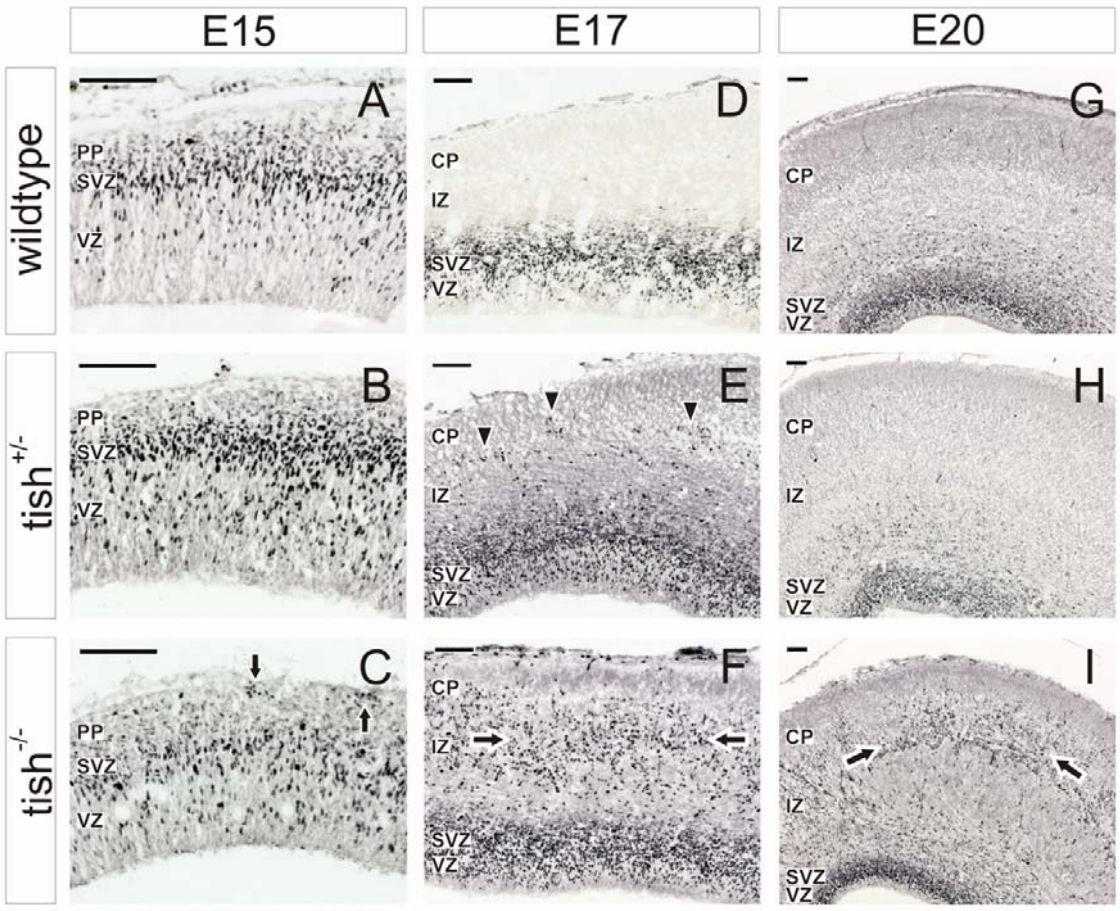



**Figure 2-2. Tbr2$^{+}$ cells are incorrectly positioned in the tish$^{-/-}$ neocortex.** Light microscopic images were taken of coronal sections of embryonic neocortex during timepoints corresponding to early, middle, and late cortical plate neurogenesis. **A-C**, Early in cortical plate neurogenesis (E15), scattered Tbr2$^{+}$ cells can be found in a superficial position in the vicinity of the preplate (PP) of the tish$^{-/-}$ neocortex (arrows). These cells are typically restricted to the developing subventricular zone (SVZ) in wildtype and tish$^{+/-}$ neocortex at this age. **D-F**, During the middle stage of cortical plate neurogenesis (E17), a diffuse band of inappropriately positioned Tbr2$^{+}$ cells can be observed in the intermediate zone (IZ) and cortical plate (CP) of tish$^{-/-}$ neocortex (arrows). These cells are normally restricted to the SVZ in wildtype and tish$^{+/-}$ neocortex. **G-I**, Late in cortical plate neurogenesis (E20), the diffuse band of inappropriately positioned Tbr2$^{+}$ cells persists in the IZ/CP of tish$^{-/-}$ neocortex (arrows). These cells are normally restricted to the SVZ in wildtype and tish$^{+/-}$ neocortex at this age. Scale bars = 100μm.

progenitors as development proceeds cannot be ruled out. This latter issue is addressed later in the thesis, and my findings are not consistent with an ongoing seeding of the heterotopic proliferative zone by normally-positioned VZ/SVZ progenitors (see Chapter 3).

In order to establish whether the abnormally located $Pax6^{+}$ or $Tbr2^{+}$ cells were, in fact, mitotic progenitors or whether they were simply post-mitotic cells that had not yet downregulated a marker of an earlier stage of development (Hevner, 2006), we employed co-labeling for Pax6 or Tbr2 and BrdU, β-III tubulin (β-tub, an early neuronal marker), and phosphorylated vimentin (pvim, a marker of mitotic cells). Cycling $Pax6^{+}$ cells (i.e., $Pax6^{+}/BrdU^{+}$ cells) were observed in an appropriate position in the VZ of wildtype, $tish^{+/-}$, and $tish^{-/-}$ neocortex at E17 (Fig. 2-3A-C). In addition, inappropriately positioned $Pax6^{+}/BrdU^{+}$ cells were present in the $tish^{-/-}$ IZ/CP, indicating that these cells were also actively engaged in the cell cycle (Fig. 2-3D and 2-3E). Similarly, mitotic $Pax6^{+}/pvim^{+}$ cells were observed at the ventricular surface in wildtype, $tish^{+/-}$, and $tish^{-/-}$ neocortices (Fig. 2-3F-H); however, double-positive cells were also found in the $tish^{-/-}$ IZ/CP, indicating that these misplaced $Pax6^{+}$ cells were undergoing mitosis (Fig. 2-3I and 2-3J).

The positioning of immature neurons relative to radial glia was examined using double labeling with Pax6 and β-III tubulin. Appropriately positioned $Pax6^{+}$/β-III $tubulin^{-}$ cells were found in the VZ of wildtype, $tish^{+/-}$, and $tish^{-/-}$ neocortices (Fig. 2-3K-M). Importantly, the misplaced $Pax6^{+}$ cells in the $tish^{-/-}$ IZ/CP were also β-III $tubulin^{-}$, indicating that these cells were indeed progenitor cells and not immature neurons (Fig. 2-

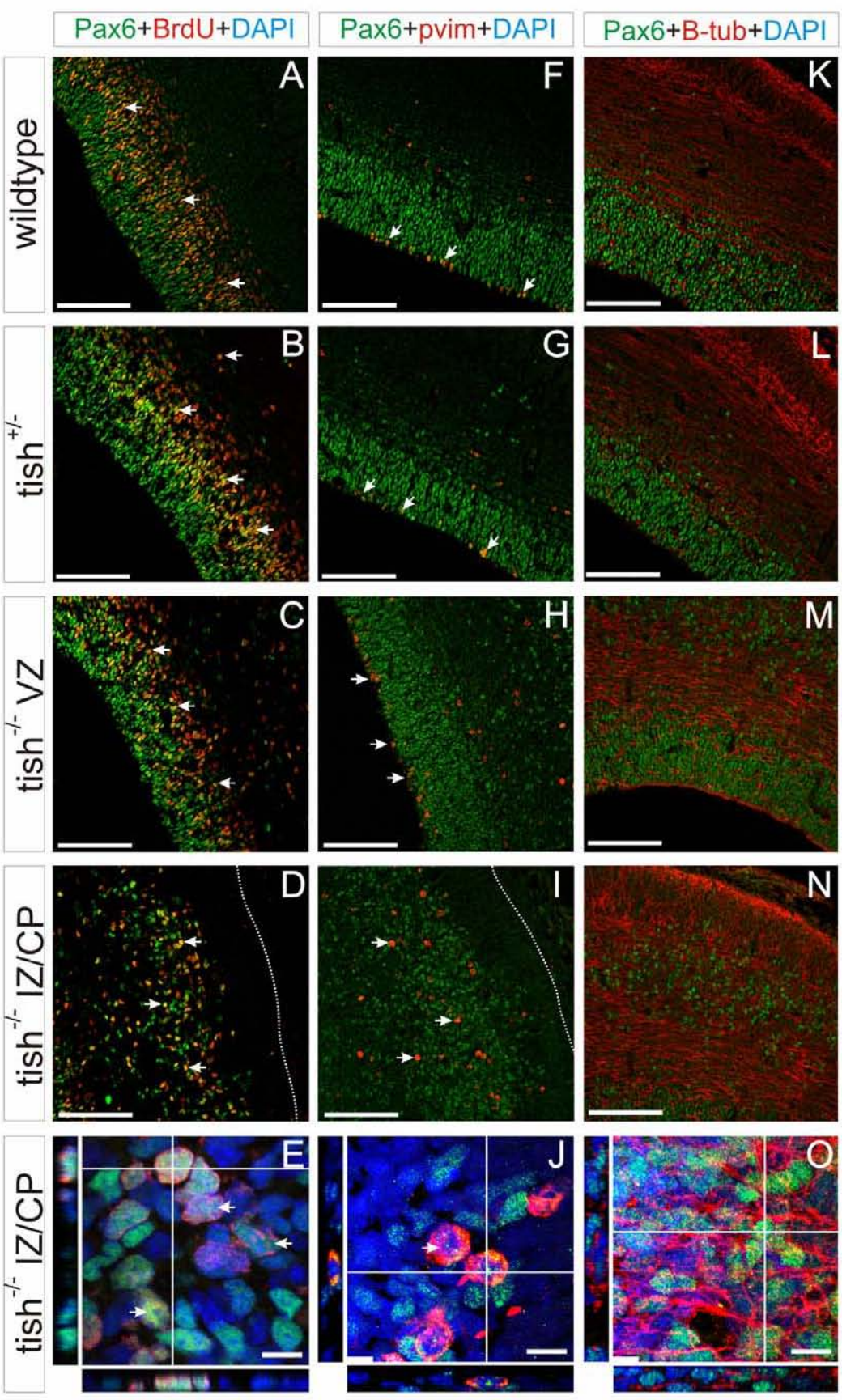

Pax6+BrdU+DAPI
Pax6+pvim+DAPI
Pax6+B-tub+DAPI
wildtype
tish+/-
tish-/- VZ
tish-/- IZ/CP
tish-/- IZ/CP
A
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**Figure 2-3. Incorrectly positioned Pax6$^{+}$ cells in the tish$^{-/-}$ neocortex are mitotic progenitor cells.** Confocal images were taken of coronal sections of embryonic neocortex at E17. In **E**, **J**, and **O**, compressed stacks are presented. Vertical and horizontal lines correspond to optical planes of section through the depth of the stack. Three-dimensional reconstructions of these regions are shown in the sidebars of **E**, **J**, and **O**. **A-E**, Actively cycling Pax6$^{+}$/BrdU$^{+}$ cells can be observed in an appropriate position in the VZ in wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices, as well as in an inappropriate position in the tish$^{-/-}$ CP (arrows indicate representative double labeled cells). **F-J**, Mitotic Pax6$^{+}$/pvim$^{+}$ cells can be found at the ventricular surface in wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices; however, Pax6$^{+}$/pvim$^{+}$ cells can also be found in the tish$^{-/-}$ CP (arrows indicate representative double labeled cells). **K-O**, Appropriately positioned Pax6$^{+}$/β-III tubulin$^{-}$ cells can be observed in the VZ of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices. Notably, the inappropriately positioned Pax6$^{+}$ cells in the tish$^{-/-}$ CP are also β-III tubulin$^{-}$ confirming that they are not immature neurons. Scale bars = 100μm in **A-D**, **F-I**, **K-N**; 10μm in **E**, **J**, **O**.

3N and 2-3O). Although these cells were located in a region of dense β-III tubulin$^{+}$ neuronal axons, three-dimensional reconstruction of confocal image stacks failed to reveal any colocalization between Pax6 and β-III tubulin. Rather, β-III tubulin$^{+}$ processes appeared to course around these Pax6$^{+}$/ β-III tubulin$^{-}$ cells (Fig. 2-3O). Interestingly, a similar phenotype was observed for the more limited population of Pax6$^{+}$ cells in the IZ of tish$^{+/-}$ neocortex, indicating that these cells were also progenitor cells and not immature neurons (Fig. 2-4A-C).

We investigated the Tbr2$^{+}$ cell population in a similar manner in order to assess the mitotic activity of these IPCs. Cycling Tbr2$^{+}$ cells (i.e., Tbr2$^{+}$/BrdU$^{+}$ cells) were observed in an appropriate position in the SVZ of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices at E17 (Fig. 2-5A-C). Misplaced Tbr2$^{+}$ cells in the tish$^{-/-}$ IZ/CP were also positive for BrdU, indicating that these inappropriately-positioned cells were actively engaged in the cell cycle (Fig. 2-5D and 2-5E). Similarly, mitotic Tbr2$^{+}$/pvim$^{+}$ cells were observed in the SVZ in wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices, consistent with the presence of a typically-positioned zone of IPCs for each genotype (Fig. 2-5F-H). Double-positive cells were also found in the tish$^{-/-}$ IZ/CP, indicating that these inappropriately located IPCs were undergoing mitosis (Fig. 2-5I and 2-5J). Finally, appropriately positioned Tbr2$^{+}$/β-III tubulin$^{-}$ cells were found in the SVZ of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices, indicative of progenitor cells and not immature neurons (Fig. 2-5K-M). The inappropriately-positioned Tbr2$^{+}$ cells in the tish$^{-/-}$ IZ/CP were β-III tubulin$^{-}$, demonstrating that they were also progenitor cells and not immature neurons (Fig. 2-5N

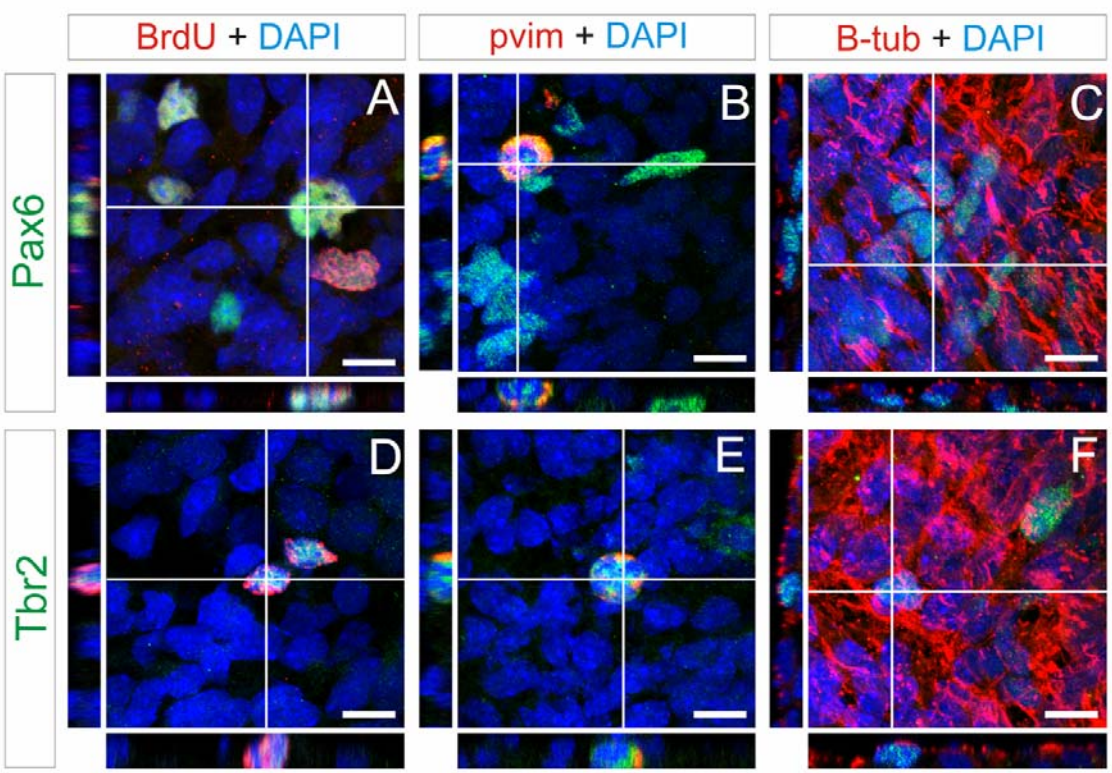


**Figure 2-4. Incorrectly positioned Pax6$^{+}$ and Tbr2$^{+}$ cells in tish$^{+/-}$ neocortex are mitotic progenitor cells.** Confocal images were taken of coronal sections of embryonic neocortex at E17, and compressed stacks are presented. Vertical and horizontal lines correspond to optical planes of section through the depth of the stack. Three-dimensional reconstructions of these regions are shown in the sidebars of each panel. **A-C**, Pax6$^{+}$ cells in the tish$^{+/-}$ IZ incorporate BrdU and are positive for p-vimentin, an M-phase marker, but they are negative for β-III tubulin, an early neuronal marker, indicating that they are proliferating progenitors and not differentiating neurons. **D-F**, Tbr2$^{+}$ cells in the tish$^{+/-}$ IZ incorporate BrdU and are positive for p-vimentin, but they are negative for β-III tubulin, an early neuronal marker, indicating that they are proliferating progenitors and not differentiating neurons. Scale bars = 10μm.

and 2-5O). While these cells were located in a region of dense β-III tubulin$^{+}$ neuronal axons, three-dimensional reconstruction of confocal image stacks failed to reveal any colocalization between Tbr2 and β-III tubulin. Rather, β-III tubulin$^{+}$ processes appeared to course around these Tbr2$^{+}$/ β-III tubulin$^{-}$ cells (Fig. 2-5O). A similar phenotype was observed for the less abundant Tbr2$^{+}$ cells in the IZ of tish$^{+/-}$ neocortex, indicating that these cells were also progenitor cells and not immature neurons (Fig. 2-4D-F). Taken together, these results indicate that a combination of mitotic radial glia and mitotic IPCs are present in the region of heterotopic proliferation in the tish$^{-/-}$ neocortex and, to a lesser extent, in a similar region in the neocortex of tish$^{+/-}$ animals as well.

**Progenitor cell density is increased in the tish$^{-/-}$ neocortex**

Qualitative observations of the Pax6 and Tbr2 immunohistochemistry results suggested that tish$^{-/-}$ neocortex contained more progenitor cells than wildtype cortex. We therefore sought to quantify these observations by measuring Pax6$^{+}$ and Tbr2$^{+}$ cell density in radial columns extending from the ventricular surface to the pial surface at each embryonic day from E15-E20. Pax6$^{+}$ and Tbr2$^{+}$ cell density across the width of the cortical wall was increased in tish$^{-/-}$ animals compared with wildtype or tish$^{+/-}$ animals beginning on E17 and proceeding through E20 (Fig. 2-6A-B). It is known that alterations in the size of the progenitor population can lead to changes in the size of the adult neocortex (Caviness et al., 2003); however, previous work from our laboratory has shown that adult tish$^{-/-}$ neocortex (normotopic cortex + SBH) is similar in volume to control neocortex (Lee et al., 1999). We therefore hypothesized that the observed increase in

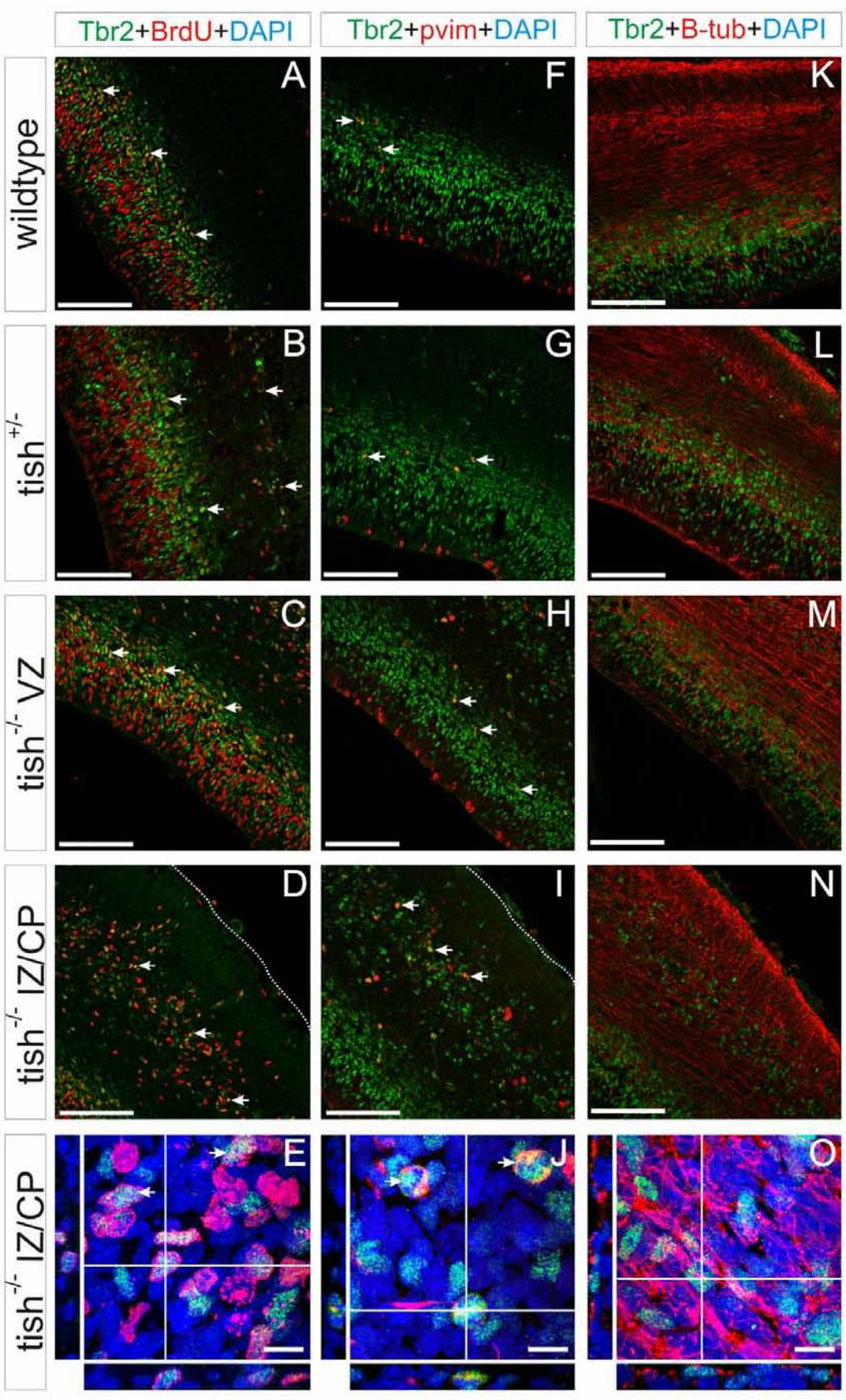
Tbr2+BrdU+DAPI
Tbr2+pvim+DAPI
Tbr2+B-tub+DAPI
wildtype
tish+/-
tish-/- VZ
tish-/- IZ/CP
tish-/- IZ/CP
A
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**Figure 2-5. Incorrectly positioned Tbr2$^{+}$ cells in the tish$^{-/-}$ neocortex are mitotic progenitor cells.** Confocal images were taken of coronal sections of embryonic neocortex at E17. In **E**, **J**, and **O**, compressed stacks are presented. Vertical and horizontal lines correspond to optical planes of section through the depth of the stack. Three-dimensional reconstructions of these regions are shown in the sidebars of **E**, **J**, and **O**. **A-E**, Actively cycling Tbr2$^{+}$/BrdU$^{+}$ cells can be observed in an appropriate position in the SVZ in wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices, as well as in an inappropriate position in the tish$^{-/-}$ CP (arrows indicate representative double labeled cells). **F-J**, Mitotic Tbr2$^{+}$/pvim$^{+}$ cells can be found at the ventricular surface in wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices; however, Tbr2$^{+}$/pvim$^{+}$ cells can also be found in the tish$^{-/-}$ CP (arrows indicate representative double labeled cells). **K-O**, Appropriately positioned Tbr2$^{+}$/β-III tubulin$^{-}$ cells can be observed in the VZ of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices. Notably, the inappropriately positioned Tbr2$^{+}$ cells in the tish$^{-/-}$ CP are also β-III tubulin$^{-}$ confirming that they are not immature neurons. Scale bars = 100μm in **A-D**, **F-I**, **K-N**; 10μm in **E**, **J**, **O**.

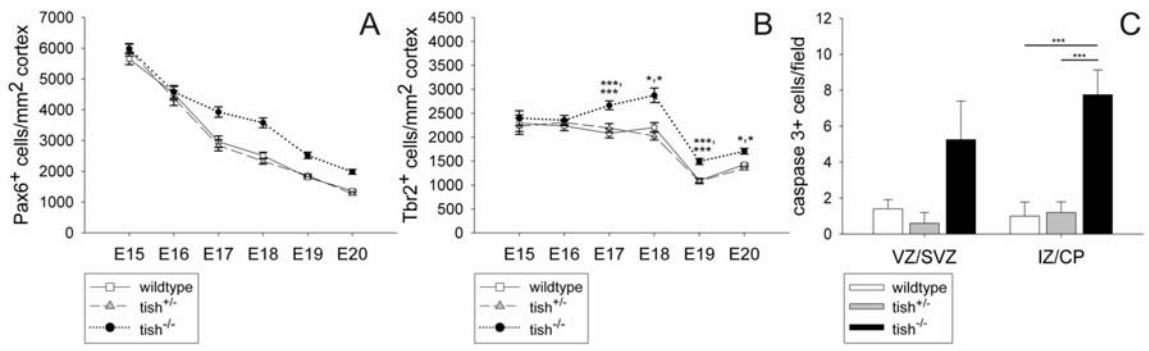



**Figure 2-6. Progenitor cell density and cellular apoptosis are increased in the tish$^{-/-}$ neocortex.** **A**, Pax6$^{+}$ cell density, measured in radial columns from the ventricular to pial surfaces, is increased in tish$^{-/-}$ neocortex as compared to wildtype and tish$^{+/-}$ neocortex beginning on E17 and persisting through E20. **B**, Tbr2$^{+}$ cell density, measured similarly, is also increased in tish$^{-/-}$ neocortex as compared to wildtype and tish$^{+/-}$ neocortex beginning on E17 and persisting through E20. **C**, Examination of activated caspase 3 positive cells in the VZ/SVZ and IZ/CP of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices revealed that there was an increased amount of cell death in the IZ/CP of tish$^{-/-}$ neocortex. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Kruskal-Wallis One-way ANOVA with Tukey's multiple comparisons testing or One-way ANOVA with Holm-Sidak multiple comparisons testing.

progenitor cell density in tish$^{-/-}$ neocortex must be offset by an increased amount of apoptosis during development. Indeed, activated caspase 3 immunohistochemistry at E17revealed a significant increase in apoptotic cell death in the IZ/CP of tish$^{-/-}$ neocortex compared with either tish$^{+/-}$ or wildtype cortex. There was also a trend toward an increase in apoptosis in the tish$^{-/-}$ VZ/SVZ (Fig. 2-6C), although this effect did not achieve statistical significance. These data indicate that, in addition to a defect in progenitor cell positioning, a disruption in progenitor cell density also occurs in the developing tish$^{-/-}$ neocortex. This alteration in cell density is offset during development by an increased level of cell death, leading to an adult neocortex whose volume is similar to that of wildtype neocortex.

Because development of the neocortex is known to proceed in a rostrolateral to caudomedial gradient (Smart and Smart, 1982), we reexamined the density of Pax6$^{+}$ and Tbr2$^{+}$ cells in the rostrocaudal and mediolateral dimensions in order to assess for any differences that might exist among wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices. For this analysis, Pax6$^{+}$ and Tbr2$^{+}$ cell density was once again quantified in radial columns from the ventricular to pial surfaces in E15-E20 embryos. Rostral sections at the level of the medial and lateral ganglionic eminences and caudal sections at the level of the developing hippocampus were analyzed. The lateral dimension was considered to be neocortex adjacent to the pallial-subpallial boundary, and the medial dimension was considered to be neocortex adjacent to the longitudinal fissure (Figure 2-7).

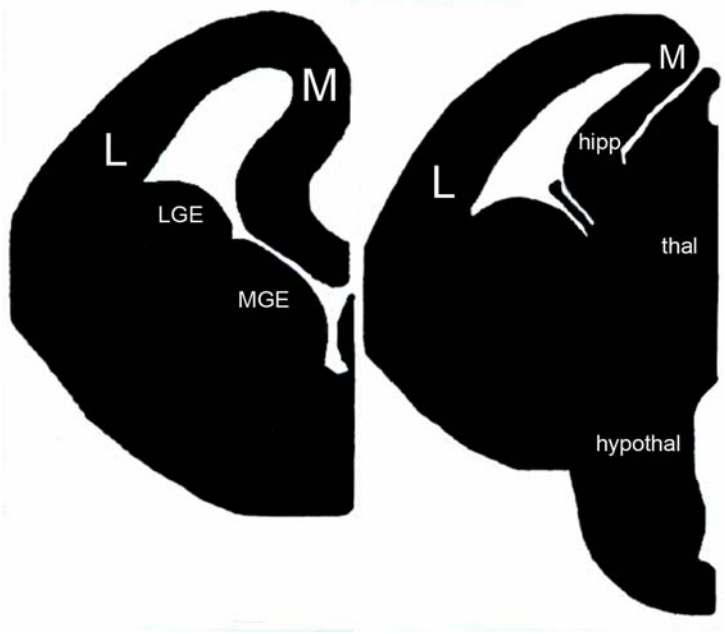
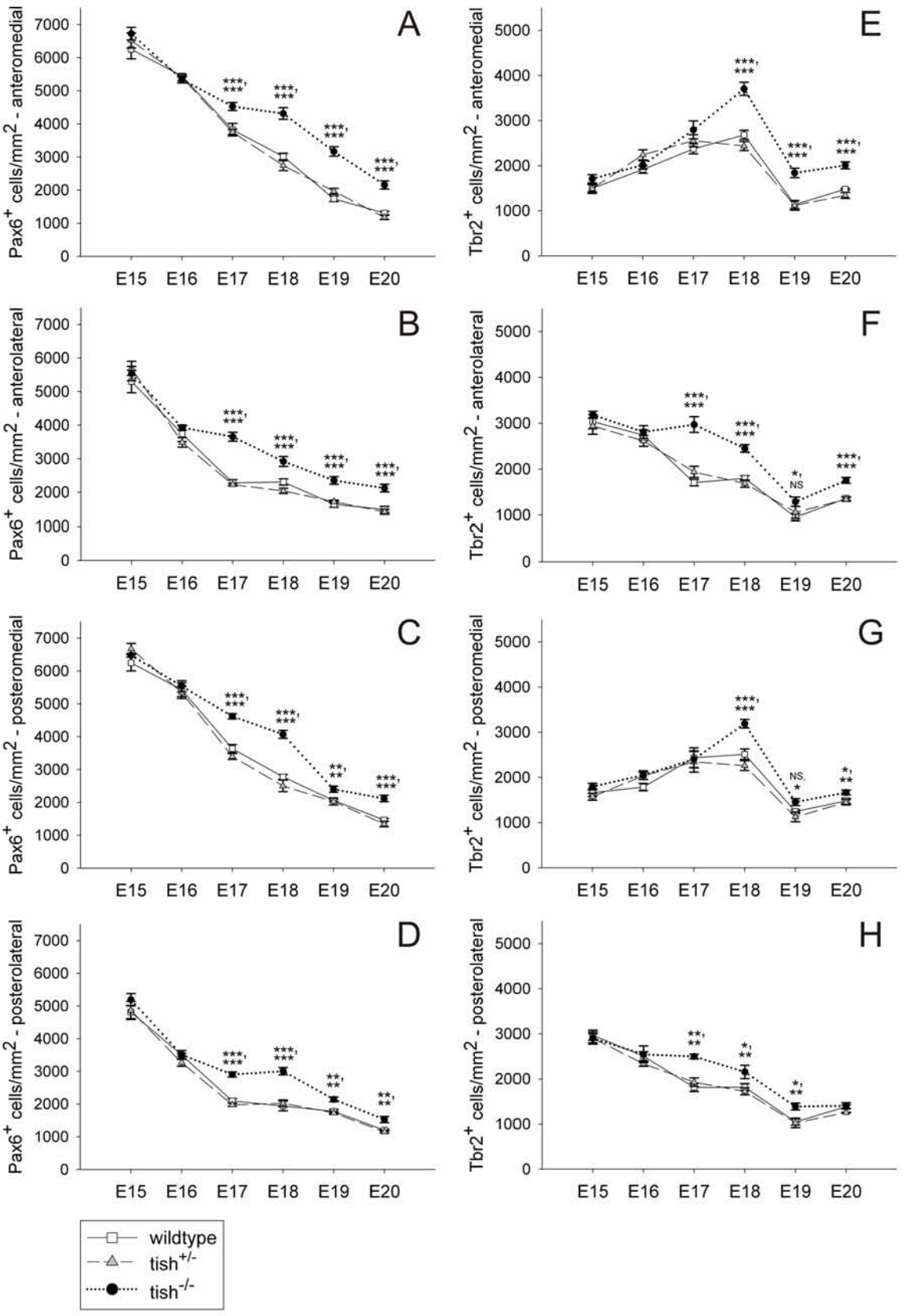



**Figure 2-7. Rostrocaudal and mediolateral measurement locations for quantification of progenitor cell density.** In order to assess for changes in progenitor cell density in tish$^{-/-}$ that might deviate from expected developmental gradients, rostral (*left*) and caudal (*right*) sections were examined in both the medial (M) and lateral (L) dimensions, and cell density was quantified as the number of cells/mm$^2$ from the ventricular surface to the pial surface at each location. *LGE*, lateral ganglionic eminence; *MGE*, medial ganglionic eminence; *hipp*, hippocampus; *thal*, thalamus; *hypothal*, hypothalamus.

With regard to $Pax6^{+}$ cell density, the results demonstrate that $tish^{-/-}$ neocortex contains a higher density of $Pax6^{+}$ cells/$mm^2$ than wildtype or $tish^{+/-}$ neocortex in all four dimensions measured (anteromedial, anterolateral, posteromedial, posterolateral) from E17-E20 (Figure 2-8A-D). Thus, there does not appear to be a developmental gradient for the disruption of $Pax6^{+}$ cell density in $tish^{-/-}$ neocortex. With regard to $Tbr2^{+}$ cell density, the results demonstrate an increase in $Tbr2^{+}$ cells/$mm^2$ in $tish^{-/-}$ neocortex compared with wildtype and $tish^{+/-}$ beginning on E17 in anterolateral and posterolateral cortex (Fig. 2-8F, H). In anterolateral neocortex, these differences persisted through E20, with the exception of a non-significant difference between $tish^{-/-}$ and $tish^{+/-}$ cortex at E19. In posterolateral neocortex, these differences persisted through E19 rather than E20. The results also demonstrate an increase in $Tbr2^{+}$ cells/$mm^2$ in $tish^{-/-}$ neocortex compared with wildtype and $tish^{+/-}$ beginning on E18 in anteromedial and posteromedial cortex (Fig. 2-8E, G). This difference persists in both locations through E20 with the exception of a non-significant difference between $tish^{-/-}$ and wildtype posteromedial cortex at E19. Thus, there is evidence for a mediolateral gradient for the disruption of $Tbr2^{+}$ cell density in $tish^{-/-}$ neocortex such that lateral areas, which are more developed than medial areas at any given embryonic age, also demonstrate a disruption in $Tbr2^{+}$ cell density that begins and ends before the disruption in the medial regions.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E
F
G
H
Pax6+ cells/mm2 - anteromedial
Pax6+ cells/mm2 - anterolateral
Pax6+ cells/mm2 - posteromedial
Pax6+ cells/mm2 - posterolateral
Tbr2+ cells/mm2 - anteromedial
Tbr2+ cells/mm2 - anterolateral
Tbr2+ cells/mm2 - posteromedial
Tbr2+ cells/mm2 - posterolateral
E15
E16
E17
E18
E19
E20
wildtype
tish+/-
tish-/-
NS

**Figure 2-8. Progenitor cell density is increased at multiple locations in the developing tish$^{-/-}$ neocortex.** **A-D**, Pax6$^{+}$ cell density was measured in radial columns from the ventricular to pial surfaces in anteromedial (**A**), anterolateral (**B**), posteromedial (**C**), and posterolateral (**D**) regions of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices from E15-E20. Pax6$^{+}$ cell density is increased in tish$^{-/-}$ neocortex as compared to wildtype and tish$^{+/-}$ beginning on E17 and persisting through E20. No developmental gradient was observed for the disruption of Pax6$^{+}$ cell density in tish$^{-/-}$ neocortex. **E-H**, Tbr2$^{+}$ cell density was measured similarly in anteromedial (**E**), anterolateral (**F**), posteromedial (**G**), and posterolateral (**H**) regions of wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices from E15-E20. Tbr2$^{+}$ cell density was increased in lateral tish$^{-/-}$ neocortex compared with wildtype and tish$^{+/-}$ beginning on E17. This difference persisted through E20 in anterolateral cortex and through E19 in posterolateral cortex. Tbr2$^{+}$ cell density was increased in medial tish$^{-/-}$ neocortex compared with wildtype and tish$^{+/-}$ beginning on E18. This difference persisted through E20 in both anteromedial and posteromedial cortex. Thus, a medial-lateral developmental gradient was observed for the disruption of Tbr2$^{+}$ cell density in tish$^{-/-}$ neocortex. Data are presented as mean ± SEM. NS non-significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, Two-way ANOVA comparing genotype and measurement location at each age for Pax6$^{+}$ and Tbr2$^{+}$ cells, with Holm-Sidak multiple comparisons test to detect differences.

**Cell cycle kinetics are altered in tish$^{+/-}$ and tish$^{-/-}$ neocortical progenitor cells**

In an effort to identify an underlying basis for the increase in progenitor cell density during mid-to-late neurogenesis in the tish$^{-/-}$ neocortex, we employed an IdU/BrdU labeling assay to assess the cell cycle kinetics of normally and abnormally positioned progenitor populations. We reasoned that, inasmuch as longer cell cycle times are associated with neurogenic rather than self-renewing divisions (Calegari and Huttner, 2003; Calegari et al., 2005), an increase in progenitor cell density at E17 could result from a shortening of the cell cycle in these populations and, thus, an increase in self-renewing divisions. We considered first the population of Pax6$^{+}$ progenitors, which correspond to radial glia. Pax6$^{+}$ cells were identified in wildtype and tish$^{+/-}$ neocortices. In addition, the tish$^{-/-}$ neocortex was further divided into assessments of the normotopic proliferative zone (n-tish$^{-/-}$) and heterotopic proliferative zone (h-tish$^{-/-}$). This latter analysis allows a differential assessment of cell cycle kinetics in the appropriately- and inappropriately-positioned proliferative cells. At E17, no significant differences were detected among groups for Pax6$^{+}$ cells in terms of the percentage of time spent in S phase ($T_s/T_c$), the total cell cycle length ($T_c$), or the lengths of S phase ($T_s$) or $G_2$+M+$G_1$ phases ($T_c$-$T_s$) (Fig. 2-9A-D, Table 2-1). In contrast, at E20, multiple differences were noted in the Pax6$^{+}$ cell populations of the various groups. Pax6$^{+}$ cells in the n-tish$^{-/-}$ neocortex did not differ from wildtype in terms of cell cycle kinetics at E20. However, E20 Pax6$^{+}$ cells in h-tish$^{-/-}$ neocortex demonstrated an increased $T_s/T_c$ compared with wildtype or n-tish$^{-/-}$ Pax6$^{+}$ cells, while Pax6$^{+}$ cells in the tish$^{+/-}$ neocortex exhibited a decreased $T_s/T_c$ (Fig. 2-9A, Table 2-1). These differences could be caused by changes in the length of S phase or

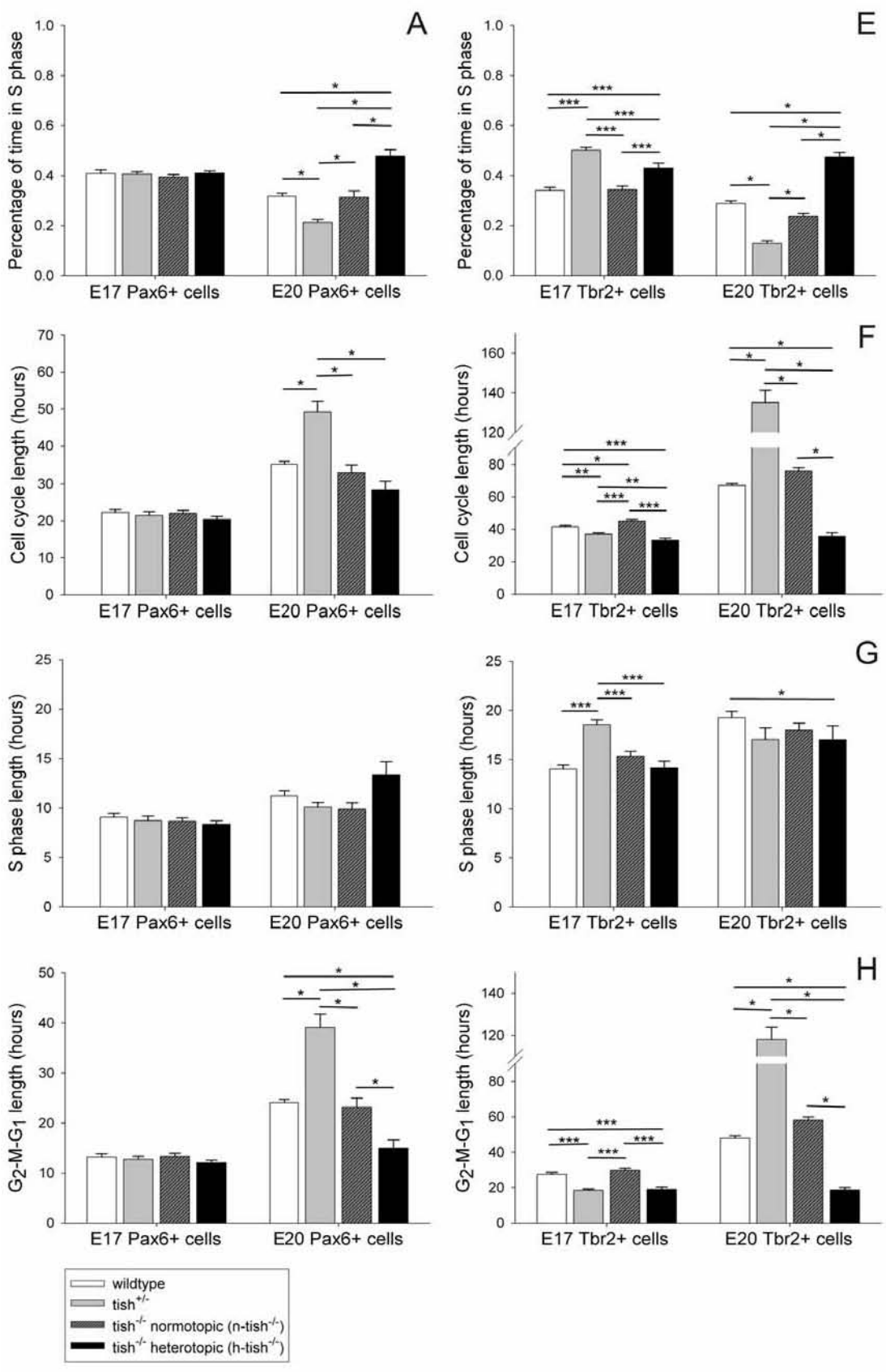
A
E
F
G
H
Percentage of time in S phase
Cell cycle length (hours)
S phase length (hours)
G2-M-G1 length (hours)
E17 Pax6+ cells
E20 Pax6+ cells
E17 Tbr2+ cells
E20 Tbr2+ cells
wildtype
tish+/-
tish-/- normotopic (n-tish-/-)
tish-/- heterotopic (h-tish-/-)

**Figure 2-9. Cell cycle kinetics are altered in tish$^{+/-}$ and tish$^{-/-}$ neocortical progenitor cells.** **A**, No differences in the percentage of time spent in S phase ($T_s/T_c$) were found among Pax6+ cells at E17 in wildtype, tish$^{+/-}$, n-tish$^{-/-}$, and h-tish$^{-/-}$ neocortices. However, at E20, h-tish$^{-/-}$ cells exhibited an increased $T_s/T_c$, while tish$^{+/-}$ cells exhibited a decreased $T_s/T_c$. **B**, Pax6$^{+}$ cell cycle length was similar among the groups at E17; however, at E20, tish$^{+/-}$ cells exhibited a longer cell cycle time than the other groups. **C**, The length of S phase of Pax6$^{+}$ cells was not significantly altered among groups at either E17 or E20, although there was a non-significant trend for a more prolonged S phase in h-tish$^{-/-}$ cells at E20. **D**, The length of $G_2$+M+$G_1$ of Pax6$^{+}$ cells was unchanged at E17; however, at E20, h-tish$^{-/-}$ cells had a significantly shorter $G_2$+M+$G_1$ length, while tish$^{+/-}$ cells exhibited an increased $G_2$+M+$G_1$ length. **E**, At E17, h-tish$^{-/-}$ Tbr2$^{+}$ cells and tish$^{+/-}$ Tbr2$^{+}$ cells each exhibited increases in $T_s/T_c$. At E20, h-tish$^{-/-}$ Tbr2$^{+}$ cells continued to demonstrate an increased $T_s/T_c$, whereas tish$^{+/-}$ Tbr2$^{+}$ cells demonstrated a significantly lower $T_s/T_c$. **F**, Tbr2$^{+}$ cell cycle length was shortened in h-tish$^{-/-}$ and tish$^{+/-}$ cells at E17. At E20, the cell cycle length was still shortened in h-tish$^{-/-}$ cells; however, it was significantly increased in tish$^{+/-}$ cells. **G**, S phase length was increased in tish$^{+/-}$ Tbr2$^{+}$ cells at E17, while it was unchanged in h-tish$^{-/-}$ cells. At E20, the length of S phase was largely unchanged among all groups except for a single significant difference between wildtype and h-tish$^{-/-}$ cells. **H**, The length of $G_2$+M+$G_1$ was significantly decreased in h-tish$^{-/-}$ and tish$^{+/-}$ Tbr2$^{+}$ cells at E17; however, at E20, h-tish$^{-/-}$ cells maintained a significantly shorter $G_2$+M+$G_1$ length, whereas tish$^{+/-}$ cells significantly lengthened their $G_2$+M+$G_1$ time. Data are presented as mean ± SEM. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, One-way ANOVA with Holm-Sidak multiple comparisons test or Kruskall-Wallis One-way ANOVA with Dunn's multiple comparisons test.

**Table 2-1.** Cell cycle parameters (mean ± SEM) for radial glial (RGC) and IPC populations in tish$^{+/-}$, normotopic tish$^{-/-}$ (n-tish$^{-/-}$), and heterotopic tish$^{-/-}$ (h-tish$^{-/-}$) proliferative zones.

| | | | $T_s/T_c$ | $T_c$ | $T_s$ | $T_s$-$T_c$ |
|---|---|---|---|---|---|---|
| RGC | E17 | wildtype | 0.41 ± 0.015 | 22.30 ± 0.78 | 9.07 ± 0.42 | 13.22 ± 0.60 |
| | | tish$^{+/-}$ | 0.41 ± 0.010 | 21.47 ± 1.05 | 8.73 ± 0.48 | 12.74 ± 0.65 |
| | | n-tish$^{-/-}$ | 0.40 ± 0.010 | 22.07 ± 0.78 | 8.67 ± 0.34 | 13.39 ± 0.55 |
| | | h-tish$^{-/-}$ | 0.41 ± 0.009 | 20.42 ± 0.82 | 8.35 ± 0.35 | 12.06 ± 0.53 |
| | E20 | wildtype | 0.31 ± 0.012 | 35.31 ± 0.81 | 11.22 ± 0.53 | 24.08 ± 0.67 |
| | | tish$^{+/-}$ | 0.21 ± 0.013 * | 49.25 ± 2.80 * | 10.12 ± 0.43 | 39.13 ± 2.63 * |
| | | n-tish$^{-/-}$ | 0.31 ± 0.026 | 33.07 ± 2.03 | 9.90 ± 0.62 | 23.17 ± 1.84 |
| | | h-tish$^{-/-}$ | 0.48 ± 0.026 * | 28.40 ± 2.35 | 13.37 ± 1.31 | 15.02 ± 1.58 * |
| IPC | E17 | wildtype | 0.34 ± 0.013 | 41.61 ± 0.89 | 14.05 ± 0.42 | 27.56 ± 1.05 |
| | | tish$^{+/-}$ | 0.50 ± 0.012 *** | 37.14 ± 0.74 ** | 18.57 ± 0.48 *** | 18.59 ± 0.65 *** |
| | | n-tish$^{-/-}$ | 0.34 ± 0.015 | 45.12 ± 1.03 * | 15.37 ± 0.49 | 29.76 ± 1.20 |
| | | h-tish$^{-/-}$ | 0.43 ± 0.019 *** | 33.28 ± 1.32 *** | 14.17 ± 0.66 | 19.11 ± 1.17 *** |
| | E20 | wildtype | 0.29 ± 0.011 | 67.20 ± 1.09 | 19.27 ± 0.63 | 47.93 ± 1.38 |
| | | tish$^{+/-}$ | 0.13 ± 0.010 * | 135.10 ± 6.20 * | 17.05 ± 1.17 | 118.05 ± 5.91 * |
| | | n-tish$^{-/-}$ | 0.24 ± 0.010 | 76.14 ± 1.83 | 18.00 ± 0.70 | 58.14 ± 1.18 |
| | | h-tish$^{-/-}$ | 0.47 ± 0.020 * | 35.79 ± 2.05 * | 17.04 ± 1.40 * | 18.75 ± 1.19 * |

**Table 2-1. Cell cycle parameters (mean ± SEM) for radial glial (RGC) and IPC cell populations in tish$^{+/-}$, normotopic tish$^{-/-}$ (n-tish$^{-/-}$), and heterotopic tish$^{-/-}$ (h-tish$^{-/-}$) proliferative zones.** An IdU/BrdU injection assay was performed on timed-pregnant dams at E17 and E20, cell counts were done on immunohistochemically processed sections, and data were analyzed within age groups for each cell population using either Kruskal-Wallis One-Way ANOVA with Dunn's multiple comparison procedure, or One-Way ANOVA with Holm-Sidak multiple comparison procedure. Significant differences are relative to wildtype. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

by a change in the length of the cell cycle caused by an alteration in the length of $G_2$+M+$G_1$. Interestingly, $T_c$ was significantly different only in tish$^{+/-}$ cells, where it was increased over all other groups. Although a trend toward a decreased $T_c$ was observed in h-tish$^{-/-}$ cells, this effect did not achieve statistical significance (Fig. 2-9B, Table 2-1). In addition, no significant differences were noted in $T_s$ among the groups, while $T_c$-$T_s$ was significantly increased in tish$^{+/-}$ cells and significantly decreased in h-tish$^{-/-}$ cells compared with wildtype cells (Fig. 2-9C-D, Table 2-1). Again, n-tish$^{-/-}$ cells did not differ from wildtype on these measures. Taken together, these results indicate that the decrease in $T_s/T_c$ in Pax6$^{+}$ cells from tish$^{+/-}$ neocortex is due to an increase in $T_c$ via a selective increase in the length of $G_2$+M+$G_1$. The increase in $T_s/T_c$ in h-tish$^{-/-}$ cells, however, is due to a decrease in the length of $G_2$+M+$G_1$ without a significant change in $T_c$. This alteration in the heterotopic cell population of proliferative radial glia provides a reasonable explanation for the increase in Pax6$^{+}$ cell density in tish$^{-/-}$ brains when one considers that a shortened cell cycle length (particularly a shortened $G_1$ phase) is associated with reentry into the cell cycle rather than differentiation. Thus, a shortened $G_2$+M+$G_1$ in heterotopic tish$^{-/-}$ progenitors suggests an increase in self-renewing divisions relative to controls, which would lead to more cells continuing to express Pax6.

In light of the cell cycle alterations in the radial glial population, we next considered the possibility that changes in cell cycle kinetics could be occurring in the IPC population. For Tbr2$^{+}$ cells at E17, $T_s/T_c$ did not differ significantly in n-tish$^{-/-}$ cells compared with wildtype. In contrast, $T_s/T_c$ was increased in tish$^{+/-}$ and h-tish$^{-/-}$ cells compared to wildtype (Fig. 2-9E, Table 2-1). These differences could be caused by

changes in the length of S phase or by a change in the length of the cell cycle due to an alteration in the length of $G_2$+M+$G_1$. Interestingly, $T_c$ was significantly decreased in both tish$^{+/-}$ and h-tish$^{-/-}$ cells compared to wildtype (Fig. 2-9F, Table 2-1). Moreover, $T_s$ was significantly increased in tish$^{+/-}$ cells, while $T_s$ did not differ significantly among the other groups (Fig. 2-9G, Table 2-1). Finally, $T_s$-$T_c$ was significantly decreased in both tish$^{+/-}$ and h-tish$^{-/-}$ cells compared to wildtype (Fig. 2-9H, Table 2-1). Taken together, these results indicate that the increase in $T_s/T_c$ in the tish$^{+/-}$ Tbr2$^{+}$ cells at E17 is due to an increase in the length of S phase along with a decrease in the length of $G_2$+M+$G_1$. In contrast, the increase in $T_s/T_c$ in h-tish$^{-/-}$ cells is produced only by alterations in $T_c$ due to a decrease in the length of $G_2$+M+$G_1$ phases.

At E20, $T_s/T_c$ in the n-tish$^{-/-}$ cortex did not differ significantly from that observed in wildtype neocortex. In contrast, $T_s/T_c$ was decreased in tish$^{+/-}$ cells and increased in h-tish$^{-/-}$ cells compared with wildtype (Fig. 2-9E, Table 2-1). $T_c$ was increased significantly in tish$^{+/-}$ cells and decreased significantly in h-tish$^{-/-}$ cells (Fig. 2-9F, Table 2-1). Moreover, $T_s$ was unchanged except for a significant decrease in h-tish$^{-/-}$ cells. The effect of these changes resulted in $T_s$-$T_c$ values that were greatly increased in tish$^{+/-}$ cells and decreased in h-tish$^{-/-}$ cells (Fig. 2-9G-H, Table 2-1). Taken together, these results indicate that the decreased $T_s/T_c$ in tish$^{+/-}$ Tbr2$^{+}$ cells and the increased $T_s/T_c$ in h-tish$^{-/-}$ Tbr2$^{+}$ cells at E20 are both due to alterations in $T_c$ via specific increases or decreases in the length of $G_2$+M+$G_1$, respectively. The alteration in cell cycle kinetics in heterotopic cells from tish$^{-/-}$ neocortex provides a reasonable explanation for the increase in Tbr2$^{+}$

cell density in tish$^{-/-}$ brains when one considers that a shortened cell cycle length (particularly a shortened $G_1$ phase) is associated with reentry into the cell cycle rather than differentiation. Thus, a shortened cell cycle length in heterotopic tish$^{-/-}$ progenitors suggests an increase in self-renewing divisions relative to controls, which would lead to more cells continuing to express Tbr2. Although IPCs are typically thought to divide terminally into two neurons, evidence indicates that these cells can also produce two proliferative daughter cells which then divide again to produce neurons (Noctor et al., 2004; Wu et al., 2005). The extent to which $G_1$ phase length predicts whether a wildtype IPC will engage in a self-renewing or differentiating division is unknown. It seems plausible, however, that heterogeneity in $G_1$ length in a population of wildtype IPCs could lead to a percentage of cells undergoing a self-renewing division prior to a terminal neurogenic division. Thus, the differences in cell cycle length observed in heterotopic tish$^{-/-}$ cells compared with wildtype could represent a further shift toward self-renewing divisions brought on by these cell cycle changes.

## Discussion

During development of the rodent neocortex, radial glia and IPCs are the principal source of glutamatergic projection neurons. After their birth in the proliferative compartments, daughter cells migrate toward the pial surface, exit the cell cycle, and initiate differentiation. While the molecular mechanisms coordinating these processes are still being elucidated, it is clear that multifunctional proteins play an important role. Indeed, the proneural factor Neurog2 is responsible for the expression of genes important for neuronal differentiation; however, it also initiates and maintains migration away from the ventricle (Miyata et al., 2004; Britz et al., 2006; Heng et al., 2008). Moreover, Neurog2 has been shown to alter the expression of other important regulators of migration, such as p35, DCX, and RhoA (Ge et al., 2006). Likewise, $p27^{kip1}$, which is important for $G_1$ arrest and cell cycle exit, has been shown to stabilize Neurog2 and inhibit RhoA signaling (Nguyen et al., 2006). In addition, $p27^{kip1}$ can be phosphorylated and stabilized by CDK5, an effector of the reelin pathway that signals to migrating neurons, and this stabilized form promotes the actin organizing function of cofilin, thus enhancing migration (Kawauchi et al., 2006). Other studies have demonstrated that CDK5 is necessary for neuronal differentiation and cell cycle inhibition, that the Cdk inhibitor $p57^{kip2}$ also has pro-migration effects, and that the cell cycle regulating retinoblastoma (Rb) protein serves another role as a controller of migration (Ferguson et al., 2002; Cicero and Herrup, 2005; Ferguson et al., 2005; Itoh et al., 2007).

Despite this coordination, these processes can be uncoupled, suggesting that cells can migrate out of proliferative compartments without exiting the cell cycle (Zindy et al., 1999; Lobjois et al., 2008). Our results demonstrate that the tish mutation disrupts the

position and cell cycle kinetics of progenitors in the developing neocortex. In light of the tight coordination of cellular positioning and movement with cell cycle behavior, we further hypothesize that the tish gene plays either a direct or indirect role in the efficient coordination of these processes. In support of this hypothesis, the studies presented herein have demonstrated that: 1) tish$^{-/-}$ neocortex, and to a lesser extent, tish$^{+/-}$ neocortex contains a population of proliferating Pax6$^{+}$ and Tbr2$^{+}$ cells in the IZ/CP, 2) these heterotopic proliferative cells are cycling progenitors and not young, differentiating neurons, 3) heterotopic progenitors are increased in their density in E17-E20 tish$^{-/-}$ neocortex, and 4) cell cycle kinetics are disrupted in both tish$^{-/-}$ and tish$^{+/-}$ progenitors.

**Dysregulation of progenitor position and density**

We have demonstrated that tish$^{-/-}$ neocortex contains mislocalized, cycling progenitors of the radial glial and IPC lineage in the IZ/CP of the developing neocortex compared with wildtype. In addition, tish$^{+/-}$ neocortex contains some mislocalized, cycling progenitors of the same lineages, albeit to a lesser degree than tish$^{-/-}$ neocortex. These abnormally-positioned cells exist early in CP neurogenesis at E15 and persist through E20. Moreover, in tish$^{-/-}$ neocortex, supernormal numbers of these progenitors are produced from E17-E20 compared with either wildtype or tish$^{+/-}$ cortex. Such a population of proliferating cells has not been reported in other animal models resulting from the mutation of neuronal migration genes. Taken together, these findings beg an important question – what role does heterotopic proliferation play in SBH formation?

It is conceivable that heterotopic progenitors might produce neurons that migrate into the SBH. Alternatively, they might exacerbate or induce aberrant neuronal migration from the VZ/SVZ by disrupting local migratory cues. In this context, it is noteworthy that the number of mislocalized progenitors affects the pathogenesis of SBH in the tish brain. Thus, although the developing tish$^{+/-}$ contains some mislocalized progenitors, these animals do not exhibit SBH or seizures as adults. Instead, only tish$^{-/-}$ animals, which generate substantially more mislocalized progenitors, demonstrate SBH with seizures as adults.

Irrespective of whether heterotopic progenitors in tish$^{-/-}$ neocortex are neurogenic or whether they disrupt otherwise normal neurogenesis from the VZ/SVZ, one component of SBH pathogenesis in this model appears to involve the positioning of guidance cues for newborn neurons. If mislocalized progenitors contribute neurons to the heterotopia, then some must necessarily migrate ventrally from their birthplace toward the heterotopia. In other words, these cells must travel away from their major migratory cue, reelin, which is produced by Cajal Retzius (CR) cells in the marginal zone immediately beneath the pial surface. Interestingly, migration of projection neurons away from the CP has been demonstrated previously in wildtype neocortex (Noctor et al., 2004). The purpose and control of such a migratory route is unclear; however, this phenomenon suggests that directional control of migration involves more than reelin secretion from CR cells. Indeed, numerous mechanisms promote radial glial survival, identity, and radial process integrity, and thus contribute to appropriate neuronal migration (Gaiano et al., 2000; Campbell, 2003; Hartfuss et al., 2003; Schmid and Anton, 2003; Yoon et al., 2008; Radakovits et al., 2009). Similarly, studies of migrating

interneurons have demonstrated the importance of progenitor-derived chemokine signaling in the coordination of migration (Tiveron et al., 2006). Other studies of radially migrating neurons have demonstrated a chemoattractive role for the diffusible factor semaphorin 3A (Chen et al., 2008). Thus, if heterotopic tish$^{-/-}$ progenitors are neurogenic, then their inappropriate local microenvironment in the period immediately after mitosis could conceivably lead to migration of daughter neurons ventrally or dorsally. It remains possible that heterotopic progenitors could also interfere with existing guidance cues or provide additional, aberrant cues to already migrating neurons from the VZ/SVZ. Such disruptions could ultimately lead to alterations in the migration and positioning of VZ/SVZ born neurons. Delineating which scenario plays a greater role in tish SBH pathogenesis is a goal for future research, both to elucidate potential mechanisms contributing to SBH formation in humans and to understand mechanisms underlying neocortical development more globally.

**Dysregulation of cell cycle kinetics**

The increase in progenitor cell density in tish$^{-/-}$ neocortex raised the possibility that the cell cycle kinetics of radial glia and IPCs are disturbed. Our findings demonstrate a shortened cell cycle length in mislocalized tish$^{-/-}$ progenitors compared with controls, which is due primarily to a shortening of $G_1$+M+$G_2$ phases. Interestingly, at E17 both normally- and abnormally-positioned radial glia in tish$^{-/-}$ neocortex behaved in a manner similar to wildtype and tish$^{+/-}$ cells. Perhaps there is a differential requirement for the tish gene in cell cycle control between radial glia and IPCs at this age. Alternatively, the role of the tish gene may be masked in this assay due to

heterogeneity among subpopulations of radial glial cells (Hartfuss et al., 2001; Pinto and Gotz, 2007). The latter possibility is more consistent with the finding that radial glial density is increased in tish$^{-/-}$ neocortex at E17.

Overall, these findings provide a rational explanation for the increase in progenitor cell density in tish$^{-/-}$ neocortex. A short cell cycle length, particularly a short $G_1$ phase, is associated with continued proliferation rather than cell cycle exit and differentiation (Calegari and Huttner, 2003; Calegari et al., 2005). Thus, the shortened $G_2$+M+$G_1$ and total cycle lengths in heterotopic tish$^{-/-}$ progenitors compared with controls suggests an increase in self-renewing divisions, which would lead to more Pax6$^{+}$ and Tbr2$^{+}$ cells. It is important to recognize that the IdU/BrdU assay in our studies does not allow the localization of differences specifically to $G_2$+M or $G_1$ as more detailed BrdU serial injection protocols would permit (Takahashi et al., 1993). Another goal for future studies would be to provide a more refined assessment of cell cycle kinetics to test $G_1$ phase length in tish$^{-/-}$ neocortex relative to controls in an effort to provide a more comprehensive explanation for the differences in progenitor cell density in tish$^{-/-}$ neocortex.

Our analysis also revealed an unexpected finding, namely, E20 radial glia and IPCs in tish$^{+/-}$ neocortex exhibited a significantly prolonged cell cycle time due to a lengthened $G_2$+M+$G_1$ period compared with controls. Moreover, E17 IPCs in tish$^{+/-}$ neocortex behaved more like mislocalized tish$^{-/-}$ cells, exhibiting a shortened cell cycle length. These results have presented a conundrum. How can haploinsufficiency for the tish gene produce a kinetic phenotype that is not intermediate to wildtype and total

insufficiency? It is known that cell cycle duration in neural progenitors lengthens as neurogenesis proceeds (Takahashi et al., 1995), but the mechanistic underpinnings of this phenomenon are not well understood. Our data suggest that two functional tish alleles are required for appropriate control over cell cycle length, that is, for progressive elongation of the cell cycle as the embryo ages. This lengthening, due to changes in $G_1$ phase, reflects a series of molecular events designed to slow and, ultimately, arrest the cell cycle, transition cells into $G_0$, and initiate differentiation. Interestingly, haploinsufficiency for the tish gene appears to result in a greatly extended timeline for the completion of these events. Presumably, an elongation of the cell cycle beyond that of wildtype would not change the cell's decision to differentiate; therefore, progenitor cell density would remain unchanged. The consequence of this change in cell cycle kinetics, however, is unknown. Ultimately, identification of the tish gene will allow the planning of future studies into its precise role in cell cycle control.

An important issue raised by our results is the question of how progenitor positioning regulates cell number. Does the tish mutation principally disturb cell positioning, with the cell cycle changes attributable to an effect of the new environment? Alternatively, does the mutation disturb both positioning and cycle regulation intrinsically, with the new environment playing either a minimal or a synergistic role? Regardless of the answer to these questions, a key issue to consider is the environmental setting of the heterotopic progenitor cells. Numerous growth factors impact neural progenitor proliferation, such as EGF, FGF-2, IGF-1, BMPs, and TGFα (Arsenijevic, 2003). Moreover, spatially-regulated exposure to notch ligands during interkinetic nuclear migration provides progenitors with an appropriate balance of proliferative and

neurogenic signals (Del Bene et al., 2008). Furthermore, gap junction coupling and purinergic signaling mediates $Ca^{2+}$ waves in radial glia and adult neural stem cells, providing a mechanism for facilitating $G_1$-S phase transitions and continued cycling in coupled progenitors (Berridge, 1995; Bittman et al., 1997; Bittman and LoTurco, 1999; Weissman et al., 2004; Mishra et al., 2006). It is intriguing to speculate that the mislocalization of a large number of $tish^{-/-}$ progenitors could disrupt critical cell-cell junctions and exposure to mitogenic signals, thus altering cell cycle kinetics.

In summary, the studies presented in this chapter have demonstrated that: 1) $tish^{-/-}$ neocortex, and to a lesser extent, $tish^{+/-}$ neocortex contains a misplaced population of proliferating $Pax6^{+}$ and $Tbr2^{+}$ cells in the IZ/CP, 2) these heterotopic proliferative cells are cycling progenitors and not young, differentiating neurons, 3) heterotopic progenitors are increased in their density in E17-E20 $tish^{-/-}$ neocortex, and 4) cell cycle kinetics are disrupted in both $tish^{-/-}$ and $tish^{+/-}$ progenitors. In light of the tight coordination of cellular positioning and movement with cell cycle behavior, these results suggest that the tish gene may play either a direct or indirect role in the efficient coordination of these processes in radial glia and intermediate progenitors.

# Chapter 3

## Cellular mechanisms underlying progenitor mislocalization in the tish neocortex

## Abstract

During neurogenesis in the pallium, neurons are born from proliferating cells in the VZ/SVZ. Subsequently, they must exit the cell cycle, initiate differentiation, and migrate along radial glial guides into the cortical plate. Upon arrival in the cortical plate, maturing neurons achieve an appropriate apical-basal polarity, extending a ramified dendrite toward the pial and an axon toward the intermediate zone, where it can join with other axons projecting to cortical and/or subcortical targets. Careful control of these processes is critical, and the failure of these mechanisms can result in proliferative or post-mitotic cells that are mislocalized or misoriented.

The tish mutation results in a population of mislocalized progenitor cells during development and, in adult tish$^{-/-}$ animals, the presence of a large structural malformation containing both neurons and glia. Therefore, studies using immunohistochemistry and *in utero* electroporation were undertaken to identify a cellular mechanism underlying the mislocalization of heterotopic progenitors and of the neurons within the SBH. The results indicated that tish$^{+/-}$ and tish$^{-/-}$ radial glia do not become mislocalized due to a loss of adherens junctions or apical polarity. Furthermore, heterotopic tish$^{-/-}$ progenitors are not produced by the pallial ventricular zone at E16.5, nor do they retain contact with the ventricular surface. Finally, the tish$^{-/-}$ VZ/SVZ produces neurons for both the cortical plate and the heterotopia. These results suggest that heterotopic tish$^{-/-}$ progenitors may fail to exit the cell cycle when they initiate migration, resulting in proliferative cells entering the IZ/CP and contributing to the heterotopic proliferative zone. They also suggest that the presence of heterotopic progenitors may distort guidance cues that

migrating neurons use to find their appropriate location in the cortex. Taken together, these findings define a form of developmental error contributing to SBH formation that differs fundamentally from a primary error in neuronal migration.

## Introduction

During the process of neocortical neurogenesis, radial glia and intermediate progenitors of the pallial proliferative zones in the VZ/SVZ produce the majority of neurons for the cortical plate via direct and indirect neurogenesis (Hevner, 2006). After their birth in the VZ/SVZ, daughter cells must arrest their cell cycles, begin differentiating, and initiate migration. Upon their arrival in the cortical plate, differentiated neurons must establish apical-basal polarity and extend an axon toward target cells. Successful completion of these processes requires a carefully choreographed series of intrinsic and extrinsic mechanisms. The failure to coordinate these processes can result in errors such as migrating, differentiating neurons that remain engaged in the cell cycle and correctly-positioned, post-mitotic neurons that orient incorrectly and project dendrites and axons in inappropriate directions (Polleux et al., 1998; Zindy et al., 1999; Polleux et al., 2000; Barnes et al., 2008; Lobjois et al., 2008).

Because tish neocortex contains a population of inappropriately-positioned, actively cycling progenitors in the IZ/CP of developing neocortex and because $Pax6^{+}$ progenitors were most severely affected, it was hypothesized that errors in the structure and/or function of radial glia might be responsible. Therefore, a series of experiments was carried out to address the following questions. What is the cellular mechanism by which heterotopic cells become mislocalized? Which proliferative zone (normotopic or heterotopic) contributes neurons to the normally-positioned cortical plate and the abnormally-positioned SBH. The broader goal of these studies is to define a form of developmental error contributing to SBH formation that differs fundamentally from a primary error in neuronal migration.

Overall, the results demonstrate that heterotopic tish$^{+/-}$ and tish$^{-/-}$ progenitors do not become mislocalized as a result of the loss of radial glial adherens junctions or polarity. Moreover, heterotopic tish$^{-/-}$ progenitors are not produced by the VZ/SVZ at E16.5, nor do they maintain a connection to the ventricular surface. Furthermore, the normally-positioned proliferative zone in tish$^{-/-}$ neocortex gives birth to neurons that migrate into both the CP and heterotopia. Those neurons that reach the CP orient correctly, extending an apical dendrite and basal axon, whereas many neurons that reach the heterotopia orient inappropriately. Taken together, these results suggest that heterotopic tish$^{-/-}$ progenitors may be unable to exit the cell cycle once they begin to migrate out of the VZ/SVZ during early neocortical neurogenesis, thus seeding a heterotopic proliferative zone upon their arrival in the IZ/CP. However, an ongoing seeding of the heterotopic proliferative zone later in neurogenesis by VZ/SVZ cells does not occur. The presence of misplaced progenitor cells below the normotopic cortex in tish$^{-/-}$ animals could disturb the local environmental cues necessary for establishing proper apical-basal orientation and migration of cells in the SBH.

## Materials and Methods

### Animals and breeding

Animals were housed at 22°C on a standard 12h:12h light–dark schedule with free access to food and water. Animals were handled according to NIH Animal Care and Use Guidelines and a protocol approved by the University of Virginia Animal Care and Use Committee. The tish phenotype is expressed on a Sprague-Dawley background, and the heterotopia are inherited in an autosomal recessive manner, requiring two mutated alleles in order to display the SBH phenotype. Therefore, timed pregnant litters of $tish^{-/-}$ pups were generated by mating a $tish^{-/-}$ male with a $tish^{-/-}$ female. Wildtype Sprague-Dawley control litters were generated by mating a wildtype male to a wildtype female. $Tish^{+/-}$ litters were generated by mating a $tish^{-/-}$ male to a wildtype female. In all cases, the morning of vaginal plug discovery was designated as embryonic day E0.5.

### Bromodeoxyuridine administration

For those animals used in the immunohistochemical characterization of tish progenitor cells, BrdU was administered as previously described (Lee et al., 1998a). Briefly, pregnant dams were given an intraperitoneal injection of BrdU (50 mg/kg, Sigma) and then euthanized 2h later under deep anesthesia. The brains of the embryos were then removed and prepared for sectioning. This administration protocol was employed to label only those progenitor cells within S phase or about to exit S phase at the time of administration, since a two-hour survival is insufficient time for these cells to complete mitosis and pass BrdU on to their progeny (Takahashi et al., 1995).

**Tissue processing and Immunohistochemistry**

Timed-pregnant dams were anesthetized with isoflurane and decapitated, and embryos were removed. Brains were rapidly dissected in 0.1M PBS and fixed for 1h in 4% paraformaldehyde, followed by cryoprotection in 30% sucrose until sinking. Cryostat sections were cut at 20 or 60μm and mounted on Superfrost Plus slides (Fisher Scientific).

Slide mounted sections were boiled briefly in 10mM sodium citrate pH 6 to enhance antigen recognition, followed by incubation in 2N HCl for 30 min to expose BrdU where appropriate. Sections were then blocked in 5% normal goat serum (Vector Laboratories, Burlingame CA) with 0.3% Triton X100 in 0.1M PBS for 2h before overnight incubation at 4˚C with primary antibody diluted in blocking serum. The following primary antibodies were used: anti-BrdU (mouse, 1:10, BD Biosciences), anti-BrdU (rat, 1:40, Abcam), anti-aPKC-λ (mouse, 1:100, BD Transduction Labs), anti-PAR3 (rabbit, 1:100, Millipore), anti-GFP (rabbit, 1:500, Invitrogen). Incubation with secondary antibody diluted in blocking serum was performed for 1h at room temperature. The following secondary antibodies were used: goat anti-rabbit Alexa 488, goat anti-mouse Alexa 594, or goat anti-rat Alexa 594 (all at 1:250, Invitrogen), followed by incubation with 4',6-diamidino-2-phenylindole (DAPI) for 5 min. For phalloidin immunohistochemistry, sections were not exposed to sodium citrate; rather, they were blocked for 20 minutes in 0.1% Triton in 0.1M PBS, followed by 10% normal goat serum in 0.1M PBS, before incubation with Alexa 488 conjugated phalloidin diluted in blocking serum (5U/mL, Invitrogen) and then DAPI. Finally, slides were air dried, coverslipped

with ProLong Gold anti-fade reagent (Invitrogen), and stored at -20˚C. Images were captured on a Zeiss LSM510 confocal microscope.

**In utero electroporation**

In order to assess the mechanisms underlying the progenitor cell mislocalization in the homozygous tish neocortex, a pCAGGS-GFP plasmid was electroporated into radial glial cells at E16.5 to allow for visualization of these cells and their progeny. Briefly, a timed-pregnant wildtype or tish$^{-/-}$ dam was anesthetized via an intraperitoneal injection of a ketamine/xylazine mixture (67/10 mg/kg) and the uterine horns were exposed via an abdominal incision. Embryos were visualized by backlighting the uterus with a fiber-optic light source, and a pulled borosilicate glass electrode (1.0mm OD/0.78mm ID, Sutter Instruments, Novato CA) containing 4mg/ml pCAGGS-GFP plasmid (a kind gift from S. Anderson) in a 0.1% solution of Fast Green dye (Sigma-Aldritch) was lowered into the lateral ventricle of the embryos and the solution was injected using an MPPI-2 pressure injector (Applied Scientific Instrumentation, Eugene OR). The plasmid was electroporated using an ECM830 square wave electroporator (BTX, Harvard Biosciences) using 5 pulses of 75V, 50ms duration, and 950ms interval. After electroporation, the dam was allowed to survive for 12 or 72h before embryos were harvested and their brains were processed for immunohistochemistry as described above.

## Results

### Radial glial adherens junctions and apical polarity domains are intact in tish$^{-/-}$ neocortex

In light of the finding that a population of radial glia and IPCs are mislocalized in the tish$^{-/-}$ neocortex, we sought to identify a mechanism by which these cells might become heterotopic. Considering that the radial glial population appeared to be affected most severely at E15 (compare Fig. 2-1C and 2-2C), we hypothesized that the observed positioning defect might result from a population of VZ radial glia losing their attachments to the ventricular surface and migrating into the IZ/CP to seed the heterotopic proliferative zone. In order to test this possibility, we employed *in utero* electroporation and immunohistochemistry techniques to assess the status of adherens junctions and apical polarity markers at the ventricular surface. We reasoned that if radial glia were losing their attachments to the ventricular surface and seeding a new proliferative zone, then we would observe disruptions in the aPKC-λ and F-actin components of VZ adherens junctions and in the apical polarity protein Par3 (Cappello et al., 2006). We also reasoned that we would observe a greater percentage of radial glia with retracted apical processes following *in utero* electroporation of a pCAGGS-GFP construct.

Examination of adherens junctions using Alexa 488 conjugated phalloidin to identify F-actin and immunofluorescence against aPKC-λ indicated no obvious differences among wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices (Fig. 3-1A-F). Had a loss of adherens junctions been responsible for the heterotopic mitoses in tish$^{+/-}$ or tish$^{-/-}$ neocortex, one would have anticipated an interruption in phalloidin and aPKC-λ

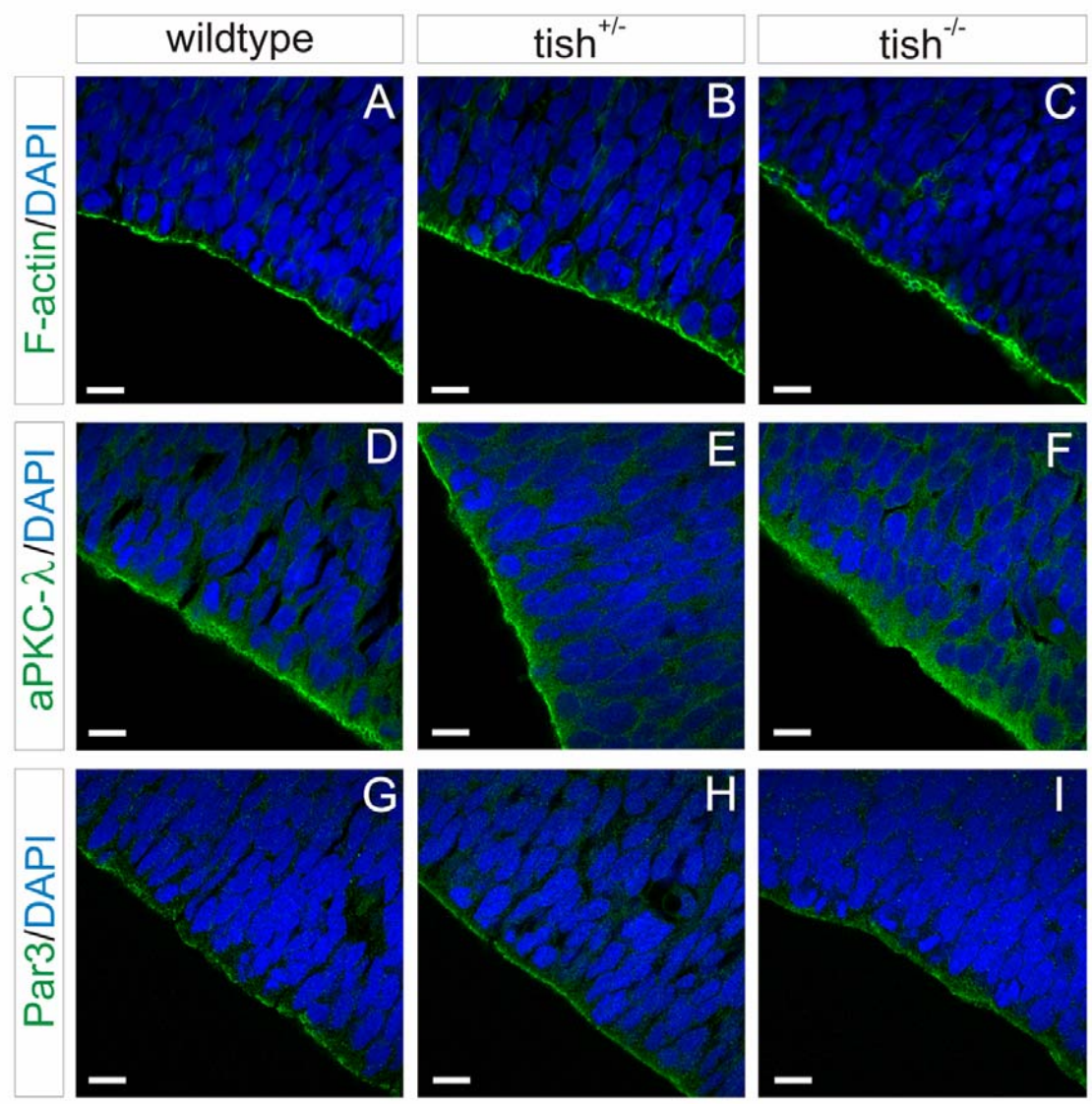


**Figure 3-1. Radial glial adherens junctions and apical polarity domains are intact at the ventricular surface of tish$^{-/-}$ neocortex.** Confocal images were taken of coronal sections of embryonic neocortex at E17. Immunohistochemistry against the adherens junction components F-actin (**A-C**) and aPKC-λ (**D-F**), and the apical polarity protein PAR3 (**G-I**) revealed no differences among wildtype, tish$^{+/-}$, and tish$^{-/-}$ neocortices, suggesting that tish$^{-/-}$ radial glia do not become mislocalized as a result of adherens junction or polarity loss. Scale bars = 10μm.

staining at the ventricular surface as has been described previously (Cappello et al., 2006). Such an interruption was not observed. Similarly, PAR3 staining revealed no obvious disruptions of apical polarity within the endfeet of radial glia at the ventricular surface (Fig. 3-1G-I). Moreover, examination of radial glial apical processes at E17, 12h after electroporation with a pCAGGS-GFP construct, revealed that the percentage of electroporated $GFP^{+}$ cells maintaining an apical process with ventricular contact did not differ between wildtype and $tish^{-/-}$ neocortex (Fig. 3-2). Thus, we conclude that adherens junctions and apical polarity within radial glial endfeet are maintained in $tish^{+/-}$ and $tish^{-/-}$ neocortex and that progenitor cells do not become mislocalized as a result of losing their apical attachments to the ventricular surface.

**Mislocalized $tish^{-/-}$ neocortical progenitor cells are not produced by the pallial ventricular zone at mid-neurogenesis**

Based on our finding that $tish^{-/-}$ radial glia maintain their ventricular attachments during a time of extensive heterotopic proliferation, it appears that a different causative mechanism underlies the mislocalization of progenitor cells in the $tish^{-/-}$ IZ/CP. One possibility is that a population of VZ radial glia could have suffered a disruption in interkinetic nuclear migration such that their nuclei failed to return to the ventricular surface to undergo mitosis. Instead, the nuclei of these cells may have continued toward the pial surface after completing S phase, dividing at some location within the IZ/CP. Alternatively, daughter cells produced by radial glial mitoses in the VZ might initiate migration and fail to undergo cell cycle arrest, instead continuing to cycle as they migrate

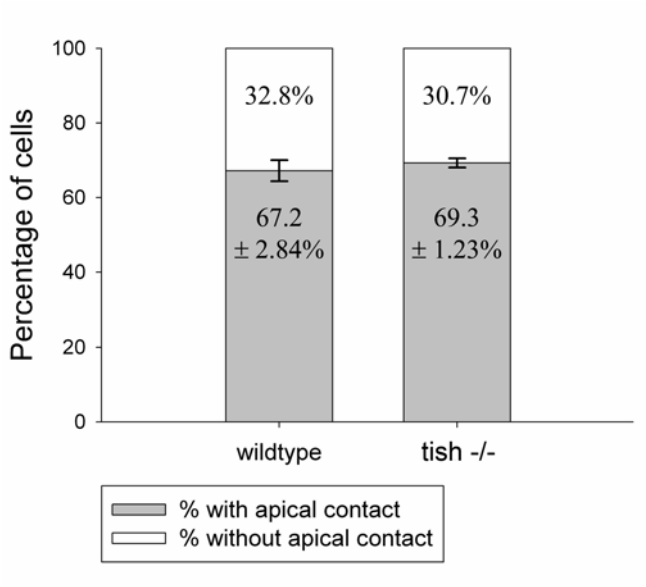


**Figure 3-2. Radial glia in the tish$^{-/-}$ ventricular zone maintain their apical processes during the middle stage of cortical plate neurogenesis.** Wildtype and tish$^{-/-}$ embryos were electroporated with a pCAGGS-GFP construct and confocal stacks were examined 12h later for the presence or absence of an apical process that contacted the ventricular surface on electroporated radial glia. The results demonstrate no difference in the percentage of radial glia with ventricle-contacting processes between wildtype and tish$^{-/-}$ neocortex, indicating that tish$^{-/-}$ radial glia do not become mislocalized secondary to the loss of their apical processes during the middle stage of cortical plate neurogenesis.

into the IZ/CP. In order to test these possibilities, we electroporated a pCAGGS-GFP construct into the lateral ventricles of E16.5 wildtype and tish$^{-/-}$ embryos and examined the neocortex 3 days later, after a single BrdU pulse 2h prior to embryo collection. We reasoned that if we found BrdU incorporating, GFP$^{+}$ nuclei in the tish$^{-/-}$ IZ/CP with apical and basal processes that spanned the width of the cortical wall, then we could conclude that an error in nuclear migration led to their mislocalization. Alternatively, if we found BrdU incorporating, GFP$^{+}$ nuclei in the tish$^{-/-}$ IZ/CP without intact ventricle-contacting processes, then we could conclude that mislocalized tish$^{-/-}$ progenitors were born from VZ radial glia, either directly or indirectly via IPCs, and that they migrated to the IZ/CP while continuing to proliferate.

In wildtype neocortex 3 days post-electroporation, GFP$^{+}$ cells were detected in the IZ and CP. Those cells in the IZ maintained a migratory morphology with a long pial-directed process characteristic of migrating neurons (Fig. 3-3A). GFP$^{+}$ cells in the CP elaborated branched apical dendrites and a basal axonal projection, which, in some cases, could be followed into the IZ (Fig. 3-3B). While there were some BrdU incorporating cells in these regions, our immunohistochemical analysis revealed no colocalization between BrdU and GFP, indicating that VZ daughter cells born at E16.5 in wildtype neocortex were able to arrest their cell cycles before migrating into the IZ.

In tish$^{-/-}$ neocortex 3 days post-electroporation, GFP$^{+}$ cells were also detected in the IZ and CP. Some cells within the IZ maintained a morphology characteristic of migrating neurons; however, other cells appeared to be stationary, extending dendrites and axons as part of the growing SBH (Fig. 3-3C and 3-4b', c', d'). Similar to wildtype,

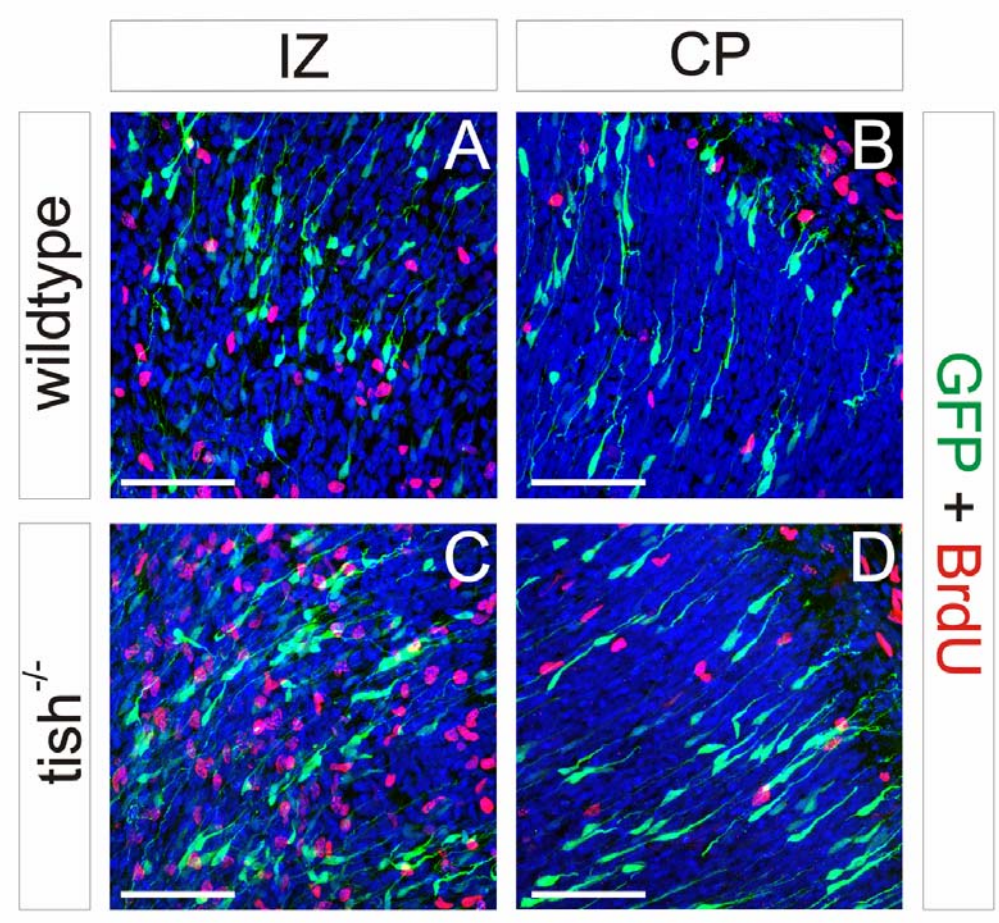


**Figure 3-3. Misplaced tish$^{-/-}$ progenitor cells are not produced by the pallial ventricular zone during the middle stage of cortical plate neurogenesis.** Wildtype and tish$^{-/-}$ embryos were electroporated with a pCAGGS-GFP construct at E16.5 and examined 3 days later, 2h after a single BrdU injection to label proliferating cells. Compressed confocal stacks of coronal sections of wildtype and tish$^{-/-}$ neocortex are presented. No GFP/BrdU colocalization was observed in the IZ (**A**) or CP (**B**) of wildtype neocortex, consistent with the idea that VZ/SVZ born cells exit the cell cycle before migrating into either location. Similarly, in tish$^{-/-}$ neocortex, no GFP/BrdU colocalization was observed in the IZ (**C**) or CP (**D**), suggesting that the heterotopic proliferative cells are not generated by the pallial VZ at E16.5. Scale bars = 50μm.

$GFP^+$ cells in the CP elaborated apical dendrites and basal axons. Surprisingly, despite the abundance of $BrdU^+$ cells in the IZ and CP of $tish^{-/-}$ neocortex, no $GFP^+$ cells were observed to incorporate BrdU (Fig. 3-3C-D, n = 3-5 sections from each of 4 embryos). These results indicate that VZ daughter cells born at E16.5 in $tish^{-/-}$ neocortex were able to arrest their cell cycles before migrating into the IZ, suggesting that VZ born cells at this age do not seed the large heterotopic proliferative zone in the IZ/CP. These data also suggest that errors in interkinetic nuclear migration at mid-neurogenesis do not underlie the progenitor cell positioning defect. Instead, heterotopic proliferative cells are either produced from a source other than the pallial VZ, or they are produced by the pallial VZ earlier in neurogenesis (before E16.5) and migrate into the IZ/CP without exiting the cell cycle, thus seeding the new proliferative zone.

**The normally-positioned proliferative zone in $tish^{-/-}$ neocortex produces neurons for both the cortical plate and heterotopia**

In light of the finding that $GFP^+$ cells were found in the CP of $tish^{-/-}$ neocortex 3 days post-electroporation, we decided to examine the distribution of electroporated cells across the cortical wall more carefully. In previous work from our laboratory, we had hypothesized that normally-positioned progenitors in the $tish^{-/-}$ neocortex produce neurons destined for the heterotopia, while abnormally-positioned progenitors produce neurons for both the CP and the heterotopia (Lee et al., 1998a). Our goal was to test this hypothesis using *in utero* electroporation at E16.5 to trace the origins of CP and SBH neurons.

In wildtype neocortex 3 days post-electroporation, $GFP^+$ cells were detected in developmental zones across the width of the neocortex and could be identified largely on the basis of morphology. $GFP^+$ cells in the VZ maintained a radial morphology with apical and basal processes characteristic of parental radial glia. $GFP^+$ cells in the SVZ possessed a multipolar morphology, indicative of neurons in phase two of radial migration, which are known to arrest in the SVZ before continuing toward the CP (Noctor et al., 2004), and IPCs (Fig. 3-4A and data not shown). Within the IZ, $GFP^+$ cells possessed a bipolar morphology with a long leading process, indicative of migrating neurons (Fig. 3-4A, a', open arrowheads). In the CP, $GFP^+$ cells were arranged in laminae beneath the pial surface, and they extended a ramified apical dendrite as well as a basal axon that could, in many cases, be traced back to the IZ (Fig. 3-4A, a", arrows).

In $tish^{-/-}$ neocortex 3 days post-electroporation, similar to wildtype, $GFP^+$ cells were detected across the width of the neocortex. $GFP^+$ cells within the VZ maintained a radial morphology with apical and basal processes characteristic of parental radial glia. $GFP^+$ cells within the SVZ possessed a multipolar morphology, indicative of either neurons in phase two of radial migration or IPCs (Fig. 3-4B and data not shown). Interestingly, $GFP^+$ cells were also located within the developing heterotopia amid the axons of the IZ and within the normally positioned CP (Fig. 3-4B, b', b"). Many of these cells in both locations could be identified as neurons based on the presence of a ramified apical dendrite and a basal axonal projection that, in some cases, could be traced into the IZ (Fig. 3-4b', b", c', c", arrows). Some $GFP^+$ neurons within the heterotopia deviated from a normal orientation, and instead these cells were oriented at angles which, in the

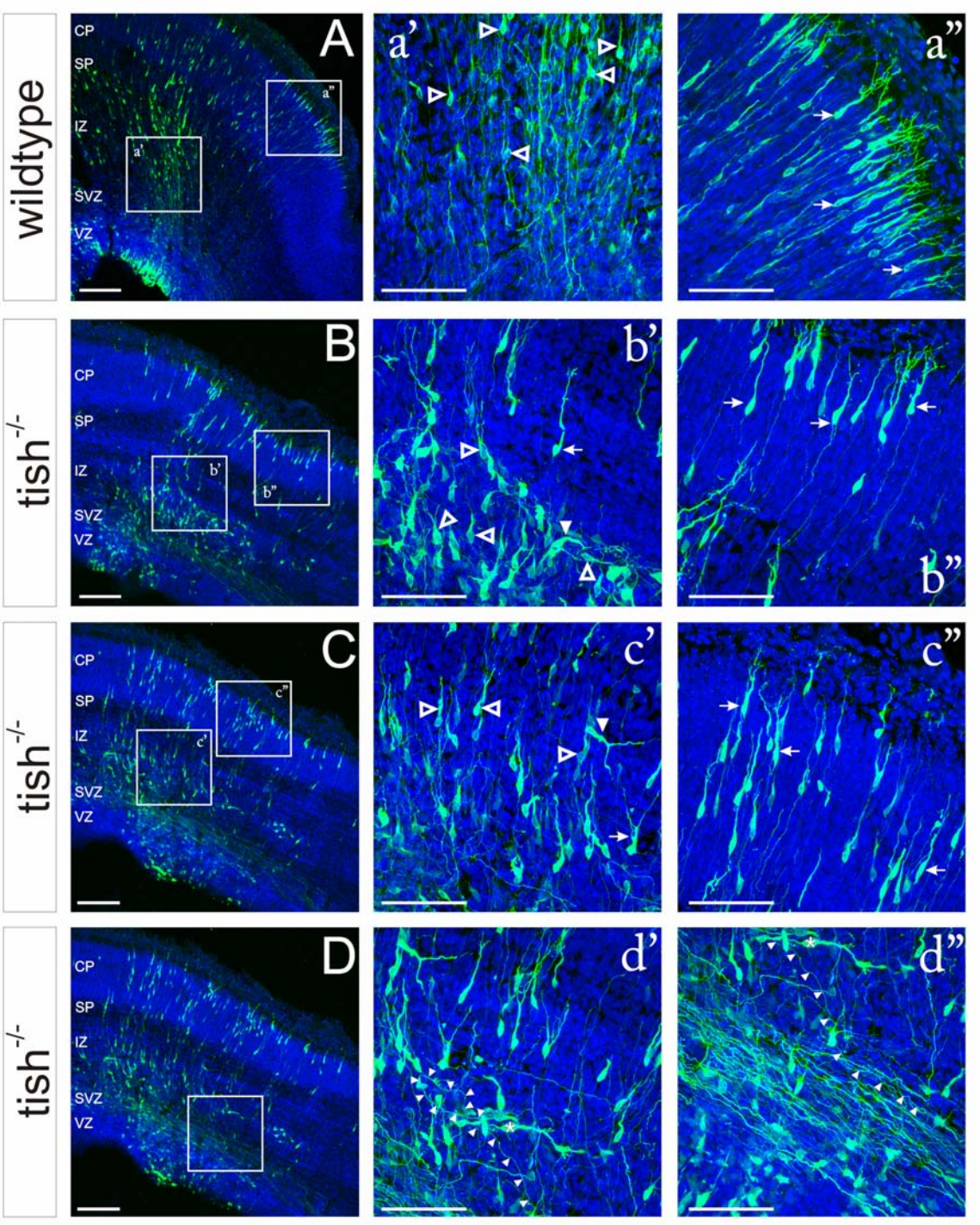


**Figure 3-4. The normally-positioned proliferative zone in tish$^{-/-}$ neocortex produces neurons for both the cortical plate and heterotopia.** Wildtype and tish$^{-/-}$ embryos were electroporated with a pCAGGS-GFP construct at E16.5 and examined 3 days later. Compressed confocal stacks of coronal sections of wildtype and tish$^{-/-}$ neocortex are presented. **A**, Wildtype neocortex 3 days post-electoporation revealed GFP$^{+}$ cells distributed across the width of the cortex, with most cells clustered in the IZ and CP. **a'**,

$GFP^+$ cells in the wildtype IZ possessed the morphology of migrating neurons (open arrowheads). **a”**, $GFP^+$ cells in the wildtype CP were primarily maturing neurons with an apical dendrite and basal axon that could, in many cases, be traced back to the IZ (arrows). **B, C, D**, $Tish^{-/-}$ neocortex 3 days post-electroporation demonstrated $GFP^+$ cells distributed across the width of the cortex, with many cells clustered in the SBH in the IZ. **b’**, **c’**, In the $tish^{-/-}$ IZ, some $GFP^+$ cells were identified as neurons within the SBH (arrows), and some neurons were misaligned at angles that deviated from their expected orientation (closed arrowheads). Other $GFP^+$ cells resembled migrating cells with a long leading process and a trailing cell body (open arrowheads). **b”**, **c”**, In the $tish^{-/-}$ CP, the majority of $GFP^+$ cells were maturing neurons that were oriented appropriately, with apical dendrites and basal axons (arrows). **d’**, **d”**, Some neurons in the $tish^{-/-}$ IZ were misaligned so severely that they were oriented parallel to the ventricular surface (* indicates soma, closed arrowheads trace an axon laterally then medially into an axon bundle, d’ and d” correspond to overlapping regions within the boxed area in D). Scale bars = 100 μm in **A-D**, 50 μm in **a’-d’**, **a”-d”**.

most severe cases, caused them to align themselves parallel to the ventricular surface (Fig. 3-4b', c', closed arrowheads). In one instance, a misaligned, heterotopic neuron was observed to project a ramified dendrite medially, while its axon coursed laterally before looping back medially and entering the white matter directed toward the contralateral hemisphere (Fig. 3-4D, d', d", * indicates cell body, closed arrowheads trace axon). Moreover, $GFP^{+}$ cells that resembled migrating cells with a long leading process and trailing cell body were observed in the heterotopia and CP (Fig. 3-4b', c', open arrowheads). In some instances, these cells appeared to be migrating as closely apposed clusters rather than as individual, spread-out entities. Taken together, these findings indicate that the normally-positioned proliferative zone produces neurons that are destined for both the normotopic CP and the heterotopia. Given that abnormally-located $tish^{-/-}$ progenitors are not generated by the VZ compartment at E16.5 and thus could not have produced neurons for the CP and heterotopia, it is most plausible to conclude that, at E16.5, normally-positioned radial glia produced daughter cells either directly or indirectly via IPCs that differentiated into neurons and migrated into both locations.

## Discussion

Based on our results that the tish mutation disrupts the position and cell cycle kinetics of progenitor cells in the developing neocortex (Chapter 2), we sought to identify a potential cellular mechanism by which these cells might become mislocalized. We focused on the radial glia of the normally-positioned VZ because $Pax6^{+}$ cells were the most severely mislocalized progenitor cell type early in neurogenesis in $tish^{-/-}$ neocortex. Also, VZ radial glia give rise, either directly or indirectly, to nearly all of the proliferative and neuronal cells of the neocortex (Anthony et al., 2004; Kowalczyk et al., 2009). We hypothesized that these pallial radial glia were responsible for generating the heterotopic progenitors throughout neocortical development. We further hypothesized that the underlying cellular mechanism(s) was a loss of adherens junction tethering at the ventricular surface, a failure of nuclei to reverse direction and return to the ventricular surface during interkinetic nuclear migration, and/or a failure of newly generated daughter cells to exit the cell cycle as they initiated migration toward the IZ/CP. The studies presented in Chapter 3 have demonstrated that: 1) apical adherens junctions and polarity are maintained in VZ radial glia in $tish^{+/-}$ and $tish^{-/-}$ neocortex, 2) heterotopic progenitors in $tish^{-/-}$ neocortex are not produced by the pallial VZ at mid-neurogenesis, nor do they maintain contact with the ventricular surface, and 3) the normally-positioned proliferative zones in $tish^{-/-}$ neocortex produce neurons for both the CP and the heterotopia.

**Tish$^{-/-}$ radial glial maintain adherens junction integrity and apical polarity**

Several previous studies have indicated that loss of function of adherens junctions leads to heterotopic mitoses throughout the depth of the neocortex with variable effects on cell cycle regulation and neurogenesis (Cappello et al., 2006; Imai et al., 2006; Lien et al., 2006). Cappello and colleagues demonstrated that conditional deletion of the small Rho-GTPase cdc42 led to normal cell cycle length despite the loss of adherens junctions and the apical localization of the Par complex and the failure of apically directed interkinetic nuclear migration (Cappello et al., 2006). Meanwhile, Imai and colleagues showed that conditional knockout of aPKC-λ caused a loss of adherens junctions, retraction of apical processes, and impaired interkinetic nuclear migration without alterations in the rate of neuron production (Imai et al., 2006). Finally, Lien and colleagues found that deletion of αE-catenin resulted in a loss of apical junctional complexes and cell polarity with shortening of the cell cycle, decreased apoptosis, and cortical hyperplasia (Lien et al., 2006). Thus, while the loss of function of adherens junctions can lead to mislocalized mitotic cells, any additional effects on cell cycle regulation or cell fate determination appears to depend on the specific component that is disrupted. It is worth noting that the presence of SBH in adulthood was not reported in any of these instances.

In light of this evidence, we reasoned that neocortical progenitors in tish$^{+/-}$ and tish$^{-/-}$ neocortex might become mislocalized via a disturbance in the structure and/or function of radial glia. Therefore, we examined the integrity of radial glial adherens junctions and apical polarity using immunohistochemistry against aPKC-λ, F-actin, and PAR3 and *in utero* electroporation of pCAGGS-GFP into radial glia. Our results indicate

that apical adherens junctions and polarity are maintained in the radial glia of tish$^{+/-}$ and tish$^{-/-}$ rats; thus, progenitor mislocalization in these animals is not related to adherens junction loss.

**Heterotopic progenitors in tish$^{-/-}$ neocortex are not produced by the pallial ventricular zone at mid-neurogenesis**

Since adherens junctions remained intact in tish$^{-/-}$ neocortex, we reasoned that heterotopic progenitors must become mislocalized by some other method that would allow the apical processes of radial glia to remain in contact with the ventricular surface. Considering that the nuclei of radial glia are normally restricted to the VZ during interkinetic nuclear migration, we reasoned that these cells must possess some mechanism for halting nuclear movement at the basal boundary of the VZ and for returning the nucleus to the ventricular surface for mitosis. It is possible that the tish mutation could disrupt some aspect of this reversal of direction, effectively uncoupling the position of the nucleus from its cell cycle stage. If this were true, one would expect to observe mitotic radial glial nuclei across the width of the neocortex that maintained apical, ventricle-contacting processes. Alternatively, since it is known that cell movement, cycle exit, and differentiation are intimately connected in wildtype neocortex, it is possible that the tish mutation could lead to an uncoupling of these processes. As a result, daughter cells born from radial glia that are properly tethered via adherens junctions would initiate migration toward the CP, but they would fail to exit the cell cycle, thus maintaining their proliferative capacity as they migrated into the IZ/CP. If this scenario were true, one would expect to find mitotic cells in the IZ/CP that did not

maintain a ventricle-contacting process. In order to test these possibilities, we employed *in utero* electroporation combined with BrdU immunohistochemistry to evaluate the morphology and proliferative capacity of electroporated cells.

Surprisingly, our results demonstrated that mislocalized tish$^{-/-}$ progenitors were not produced by the pallial VZ during mid-neurogenesis at E16.5. Inasmuch as no GFP$^{+}$/BrdU$^{+}$ cells were observed in tish$^{-/-}$ IZ/CP, we could not directly assess the morphology of heterotopic progenitors. However, the lack of any observable GFP$^{+}$/BrdU$^{+}$ cells strongly suggests that these cells do not retain contact to the lateral ventricle. Rather, it is more parsimonious to conclude either that heterotopic tish$^{-/-}$ progenitors are born from a source other than the pallial VZ/SVZ or, more likely, that they originate from the pallial VZ/SVZ prior to E16.5, migrate into the IZ/CP, and continue cycling instead of differentiating. Future studies will need to employ *in utero* electroporation at earlier ages to investigate these possibilities.

Since it is unknown how long heterotopic progenitors can self-renew before differentiation, we had anticipated that the VZ/SVZ proliferative zones would need to seed the heterotopic proliferative zone throughout neurogenesis. Assuming that heterotopic progenitors are produced by the pallial VZ earlier in neurogenesis (before E16.5), the present results suggest that this new proliferative zone maintains enough capacity for self-renewal to allow it to persist and even expand independent of a contribution from the VZ/SVZ. This possibility is not necessarily surprising, considering that radial glia are the primary self-renewing progenitor type in the developing pallium and that at least some heterotopic progenitors maintain radial glial character, as evidenced by their expression of Pax6 (Noctor et al., 2004).

It is important to recognize that our results cannot exclude the possibility that heterotopic progenitors in tish$^{+/-}$ and tish$^{-/-}$ neocortex may come from a non-pallial source. Presently, it is thought that glutamatergic neurons originate from progenitors of the pallial VZ/SVZ (Anthony et al., 2004; Kowalczyk et al., 2009). Other neocortical cell types, however, have been shown to originate from non-pallial sources. Interneurons destined for the neocortex are born in the subpallial medial (MGE) and caudal (CGE) ganglionic eminences and in the preoptic area (POA), after which they embark on a lengthy tangential migration into the neocortex (Marin and Rubenstein, 2001; Xu et al., 2004; Gelman et al., 2009). Cajal Retzius (CR) cells, the reelin-secreting cells of the pallial marginal zone, originate from multiple sites, including the pallium and also non-pallial sites such as the caudomedial wall of the telencephalic vesicles, the cortical hem, the pallial-subpallial boundary, and the septum (Hevner et al., 2003; Takiguchi-Hayashi et al., 2004; Bielle et al., 2005). Oligodendrocyte precursors have been shown to originate from subpallial sources and migrate into the pallial SVZ, while astrocytes are generated either from subpallial-derived progenitors that migrate into the pallial SVZ or from pallial radial glia (Voigt, 1989; Woodruff et al., 2001; Marshall and Goldman, 2002).

It is unlikely that heterotopic tish$^{+/-}$ and tish$^{-/-}$ progenitors are interneuron precursors that originated in the MGE, CGE, or POA. Proliferative cells have not been reported to migrate out of the VZ/SVZ of these regions, nor do they express markers characteristic of glutamatergic precursors (Pax6 and Tbr2) as heterotopic progenitors do. Instead, interneuron precursors express some combination of Nkx2-1, Nkx2-2, Nkx6-2, Dlx2, Lhx6, and Lhx7 (Flames et al., 2007). It is also unlikely that heterotopic tish$^{+/-}$ and

tish$^{-/-}$ progenitors are CR cells, as previous studies have indicated that reelin expression is restricted to the marginal zone in tish$^{-/-}$ neocortex and is not expressed heterotopically (Lee et al., 1998a).

It is possible that heterotopic tish$^{+/-}$ and tish$^{-/-}$ progenitors could be a population of proliferating oligodendrocyte or astrocyte precursors that migrated into the IZ/CP of the neocortex instead of the SVZ. However, oligodendrocyte precursors are known for their expression of Olig1, Olig2, Sox10, and PDGFR-α, not Pax6 or Tbr2, which are thought to be part of a transcription factor cascade in glutamatergic neurogenesis in the neocortex, hippocampus, and cerebellum (Zhou et al., 2000; Hevner et al., 2006). Astrocyte precursors can express some combination of Pax2, A2B5, or CD44 (Liu and Rao, 2004), and, insofar as pallial radial glia are known to produce astrocytes, Pax6 as well. Based on their marker expression, it is possible that heterotopic Pax6$^{+}$ progenitors could be astrocyte precursors; however, since Tbr2 is a marker of committed neuronal progenitors, it is unlikely that heterotopic Tbr2$^{+}$ progenitors would produce astrocytes. Therefore, the most parsimonious classification of heterotopic progenitors, based on their marker expression and positioning, is that they are neuronal (or perhaps astrocytic) progenitors originating from the pallium at some time early in cortical plate genesis. Future experiments, using a panel of markers indicative of neuronal, astrocytic, and oligodendrocytic precursors, will be needed to more definitively establish the competence of heterotopic tish$^{+/-}$ and tish$^{-/-}$ progenitors for producing daughter cells of the various lineages.

**The normally-positioned proliferative zone in tish$^{-/-}$ neocortex produces neurons for both the cortical plate and heterotopia**

The initial hypothesis for the formation of tish$^{-/-}$ SBH stated that normally-positioned progenitors contributed neurons to the heterotopia, while abnormally-positioned progenitors produced neurons for both the CP and the heterotopia (Lee et al., 1998a). Support for this hypothesis came from BrdU birthdating studies which indicated that the SBH is created in a rim-to-core fashion, suggesting that neurons may be added from both the dorsal and ventral direction (Lee et al., 1997). Such a hypothesis necessitates that some neurons migrate ventrally from the heterotopic proliferative zone toward the heterotopia rather than toward the CP. In order to test this hypothesis, we utilized *in utero* electroporation at E16.5 to trace the origins of CP and SBH neurons. Our results indicate that the normally-positioned proliferative zone in the VZ/SVZ of tish$^{-/-}$ neocortex produces neurons that migrate into both the CP and the heterotopia.

Inasmuch as no electroporated progenitors were observed in the tish$^{-/-}$ IZ/CP, we concluded that electroporated neurons in the SBH must have originated in the VZ/SVZ at E16.5 rather than from proliferating cells in the IZ/CP. However, this finding does not exclude the possibility that heterotopic tish$^{-/-}$ progenitors born at time points earlier in cortical plate neurogenesis, that is, prior to E16.5, might contribute neurons to either the CP or heterotopia. Future experiments employing *in utero* electroporation at earlier timepoints will be required to clarify this possibility. While this strategy may establish a role for heterotopic tish$^{-/-}$ progenitors in producing SBH neurons, it is possible that abnormally-positioned tish$^{-/-}$ progenitors might arise from a non-pallial origin that is not

amenable to labeling via *in utero* electroporation. Should this be the case, transgenic technologies have proven useful for tracing the lineage of neocortical neurons, and focal electroporation has been used to follow migrating interneurons from their birthplace in the ganglionic eminences to their final destination in the CP (Stuhmer et al., 2002; Anthony et al., 2004). Thus, either approach may be of benefit for assessing the contribution of the heterotopic tish$^{-/-}$ proliferative zone to the SBH.

With regard to electroporated cells in the tish$^{-/-}$ SBH, many neurons were observed to deviate from their normal orientation. In the most severe case, a misaligned, heterotopic neuron was observed to project a ramified dendrite medially, while its axon coursed laterally before looping back medially and entering the white matter directed toward the contralateral hemisphere. The observation that SBH neurons born in the tish$^{-/-}$ VZ/SVZ were unable to maintain a proper apical-basal alignment suggests that tish$^{-/-}$ neocortex possesses disruptions in the environmental cues necessary for appropriate neuronal orientation. Evidence suggests that semaphorin 3A gradients in the cortical plate and functional neuropilin 1 receptors play important roles in attracting developing dendrites toward and directing axons away from the pial surface, thus ensuring proper apical-basal orientation (Polleux et al., 1998; Polleux et al., 2000; Barnes et al., 2008). Interestingly, heterotopic neurons in the tish$^{-/-}$ SBH retain the ability to project to appropriate subcortical targets; therefore, axon guidance cues appear to be intact in tish$^{-/-}$ neocortex (Lee et al., 1997; Schottler et al., 1998; Schottler et al., 2001). Future experiments directed toward delineating any alterations in diffusible factors such as

semaphorin 3A would be an important first step in understanding the mechanistic underpinnings of misoriented neurons in the tish$^{-/-}$ neocortex.

In summary, the studies presented in Chapter 3 have demonstrated that: 1) apical adherens junctions and polarity are maintained in VZ radial glia in tish$^{+/-}$ and tish$^{-/-}$ neocortex, 2) heterotopic progenitors in tish$^{-/-}$ neocortex are not produced by the pallial VZ at mid-neurogenesis, nor do they maintain contact with the ventricular surface, and 3) the normally-positioned proliferative zones in tish$^{-/-}$ neocortex produce neurons for both the normotopic CP and the heterotopia. These results suggest that heterotopic tish$^{-/-}$ progenitors are born from a source other than the pallial VZ/SVZ or, more likely, that they originate from the pallial VZ/SVZ prior to E16.5, migrate into the IZ/CP, and continue cycling instead of differentiating.

# Chapter 4

## Conclusions and Implications

## Overview

One of the major categories of cortical malformation, classical lissencephaly, occurs at a frequency of at least 1 in 100,000 live births, with the frequency likely closer to 1:13,000 or 1:20,000 (Dobyns et al., 1993). Patients with classical lissencephaly exhibit mental retardation and epilepsy due to several different brain pathologies, namely agyria, pachygyria, or subcortical band heterotopia. Due to the size and severity of the neocortical malformation, surgical intervention is not feasible, and in many instances, the associated seizures are refractory to medication (Guerrini et al., 2008). The paucity of effective treatment modalities makes this cortical malformation syndrome an attractive candidate for research. A better understanding of the mechanistic underpinnings of classical lissencephaly could lend itself to the development of more rational management strategies and treatment modalities for the affected individuals.

Presently, research into the genetic causes and cellular mechanisms underlying classical lissencephaly has resulted in its classification as a neuronal migration disorder. Despite this substantial effort, there exist many human cases for which no causative gene or mechanism has been identified, leaving open the possibility for alternative mechanisms of this disorder (Leventer, 2005; Delatycki and Leventer, 2009). The findings presented in this thesis define a form of developmental error contributing to the formation of subcortical band heterotopia (SBH) that differs fundamentally from a primary error in neuronal migration. A broader goal of this research is to elucidate causative mechanisms for SBH formation that may be beneficial in understanding human cases of classical lissencephaly whose cellular mechanisms remain elusive. Specifically, these experiments utilizing the seizure-prone tish rat demonstrate that a population of

mislocalized progenitor cells in the developing neocortex contributes to the formation of SBH. Moreover, these experiments suggest that daughter cells born from neocortical radial glia in the tish$^{-/-}$ neocortex may be unable to exit the cell cycle after birth. Therefore, they continue to cycle as they migrate into the intermediate zone and cortical plate, thus seeding the heterotopic proliferative zone that contributes to SBH formation.

## The role of heterotopic progenitor cells in SBH formation

During cortical plate neurogenesis in wildtype neocortex, neurons are produced by VZ or SVZ progenitors by one of two processes, direct or indirect neurogenesis (Fig. 1-3 and Fig. 4-1, top row). During direct neurogenesis, proliferative radial glia undergo mitosis at the ventricular surface of the VZ. The newly generated daughter cell then begins migrating toward the CP using the parental radial glial fiber, or the radial fiber of an adjacent radial glial cell. As it migrates, this daughter cell must transition from the cell cycle into a post-mitotic, $G_0$ state, while initiating neuronal differentiation programs. Upon its arrival in the CP, the immature neuron undergoes a maturation process, elaborating a ramified apical dendrite and a basal axon, which projects to other cortical or subcortical targets (Fig. 4-1, top left panel). During indirect neurogenesis, proliferative radial glia divide at the ventricular surface of the VZ, but the daughter cell does not undergo cell cycle arrest as it begins to migrate. Instead, this intermediate progenitor cell (IPC) continues to cycle and takes up residence in the SVZ. At its next division, an IPC usually divides symmetrically to produce two cells which exit the cell cycle and initiate neuronal differentiation programs as they migrate into the CP (Fig. 4-1, top right panel). In some instances, an IPC can divide symmetrically to produce two more IPCs, which

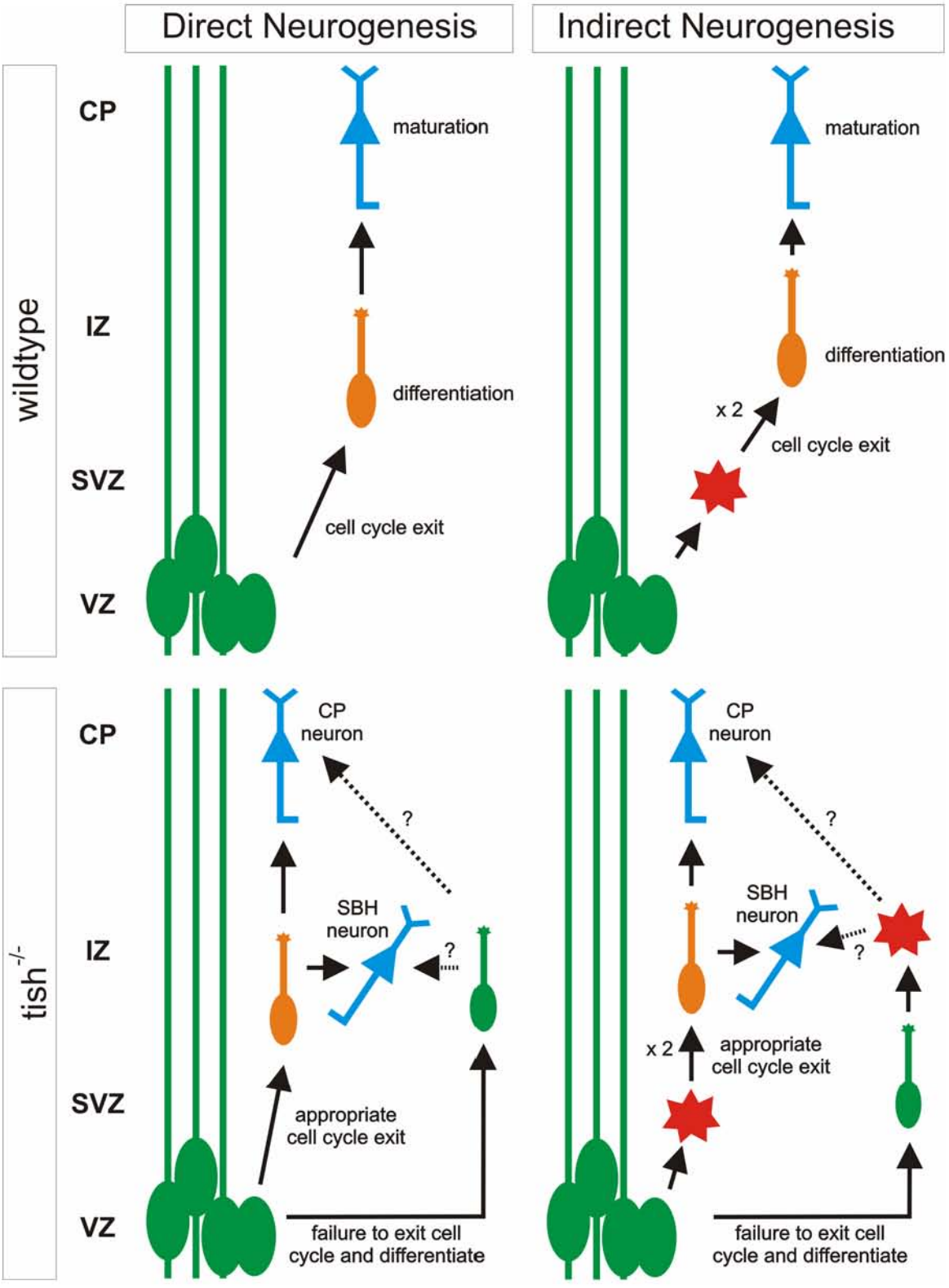
Direct Neurogenesis
Indirect Neurogenesis
wildtype
CP
IZ
SVZ
VZ
maturation
differentiation
cell cycle exit
x 2
tish-/-
CP neuron
SBH neuron
?
appropriate cell cycle exit
failure to exit cell cycle and differentiate

**Figure 4-1. Model depicting the effect of the tish mutation on progenitor cell positioning during direct and indirect neurogenesis**. **Top left,** During direct neurogenesis in wildtype neocortex, $Pax6^{+}$ radial glia (green), which are tethered to one another via adherens junctions, divide in an apical position adjacent to the ventricular surface. This division produces a daughter cell which exits the cell cycle, migrates toward the CP, and initiates differentiation (yellow). Upon arrival in the CP, differentiating cells extend a ramified apical dendrite and basal axon and finish maturation (blue). **Top right,** During indirect neurogenesis in wildtype neocortex, $Pax6^{+}$ radial glia (green) give rise to $Tbr2^{+}$ intermediate progenitor cells (red), which, upon arrival in the SVZ, divide symmetrically to produce two daughter cells. These daughters exit the cell cycle, migrate into the IZ, and initiate differentiation (yellow). Once they reach the CP, these immature neurons extend a ramified apical dendrite and basal axon and finish maturation. **Bottom left**, During direct neurogenesis in the $tish^{-/-}$ neocortex, $Pax6^{+}$ radial glia (green), which remain tethered to one another in the VZ, may give rise to daughter cells which appropriately exit the cell cycle, migrate into the IZ, and initiate differentiation (yellow). Based on our results, some of these immature neurons continue into the CP and orient properly, while others become arrested in the IZ and form the SBH. However, some daughter cells of VZ radial glial divisions may fail to exit the cell cycle and properly initiate differentiation, migrating into the IZ/CP while continuing to cycle and express Pax6. These cells are hypothesized to seed the heterotopic $tish^{-/-}$ proliferative zone and may also contribute neurons to the CP and heterotopia. **Bottom right,** During indirect neurogenesis in the $tish^{-/-}$ neocortex, $Pax6^{+}$ radial glia (green), which remain tethered to one another in the VZ, may give rise to $Tbr2^{+}$ intermediate progenitor cells (red), which, after migrating into the SVZ, divide symmetrically to produce two daughter cells. These daughters appropriately exit the cell cycle, migrate into the IZ, and initiate differentiation (yellow). Based on our data, some of these immature neurons continue into the CP and orient properly, while others become arrested in the IZ and form the SBH. However, some daughter cells of VZ radial glial divisions may fail to exit the cell cycle and properly initiate differentiation, migrating into the IZ/CP while continuing to cycle and express Pax6. These cells may then give rise to heterotopic $Tbr2^{+}$ intermediate progenitors, which are hypothesized to seed the heterotopic $tish^{-/-}$ proliferative zone and may also contribute neurons to the CP and heterotopia.

divide again to produce two neurons each (Noctor et al., 2004) (not shown in Fig. 4-1 for the sake of simplicity). There is some debate as to which mechanism is more prevalent during cortical plate formation, though more recent studies indicate that indirect neurogenesis may dominate throughout cortical neurogenesis (Kowalczyk et al., 2009).

The studies presented in this thesis using the tish neurological mutant demonstrate that a population of proliferating radial glia and intermediate progenitor cells in the IZ/CP can influence SBH formation. Specifically, it was shown that a small number of heterotopic progenitors was not sufficient to establish adult SBH in the tish$^{+/-}$ neocortex. However, when substantially greater numbers of heterotopic progenitor cells were present, adult SBH were established, as seen in the tish$^{-/-}$ neocortex. It is unclear, based on our results, whether mislocalized tish$^{-/-}$ progenitors are neurogenic progenitors of pallial VZ origin, or whether they might originate elsewhere and/or produce a cell type other than neurons. It is known that neuronal progenitors of the pallium, hippocampus, and cerebellum express the transcription factors Pax6 and Tbr2 as part of a conserved genetic program during glutamatergic neurogenesis (Hevner et al., 2006). Because heterotopic progenitors express Pax6 and Tbr2, much like glutamatergic neuronal progenitors of the pallial VZ/SVZ (Fig. 2-1 through 2-5), the most parsimonious interpretation is that heterotopic progenitors originate from pallial radial glia and retain their proliferative ability as they migrate toward the CP.

What effect might these heterotopic progenitors have on direct and indirect neurogenesis from the VZ/SVZ? Immunohistochemical studies of radial glial adherens junctions and apical polarity demonstrated that VZ radial glia in tish$^{+/-}$ and tish$^{-/-}$ neocortex maintain appropriate polarity and adherens junction tethering (Fig. 3-1 and 3-

2). This finding not only demonstrates that heterotopic progenitors are not simply radial glia that detach from the ventricular surface but also that the structural integrity of the tish VZ is maintained as in wildtype. The results from the *in utero* electroporation studies of tish$^{-/-}$ neocortex reveal that daughter cells born from pallial radial glia are capable of differentiating and migrating into both the CP and the heterotopia (Fig. 3-4). Therefore, some proportion of daughter cells from VZ/SVZ tish$^{-/-}$ progenitors produced via direct and indirect neurogenesis must be capable of exiting the cell cycle and differentiating, as in wildtype neocortex (Fig. 4-1, bottom panels). Taken together, these results imply that, if heterotopic progenitors were to originate from the pallial VZ, then some radial glial daughter cells born at the ventricular surface must fail to properly exit the cell cycle and initiate differentiation as they migrate (Fig. 4-1, bottom panels). In support of the concept that the tish mutation results in errors in cell cycle regulation, the results of our cell density measurements and a cell cycle kinetics assay demonstrated that tish$^{-/-}$ neocortex contains supernumerary progenitor cells and that their cell cycle kinetics are disturbed (Fig. 2-6, 2-8, 2-9). While the results of our *in utero* electroporation studies do not directly demonstrate that some tish$^{-/-}$ cells fail to exit the cell cycle properly, they do indicate that heterotopic progenitors are not generated anew at the middle stages of cortical plate neurogenesis, nor do the already existing cells in the IZ/CP retain contact with the ventricular surface (Fig. 3-3). Thus, the most parsimonious interpretation of these data is that either heterotopic progenitors in tish$^{-/-}$ neocortex are not produced by the pallial proliferative zone at any stage or, more likely, that they are produced by the pallial VZ/SVZ prior to the middle stages of cortical plate neurogenesis, and they retain the capacity for self-renewal throughout development.

While the model of direct and indirect neurogenesis for tish$^{-/-}$ neocortex presented in Figure 4-1 is consistent with the results of the studies presented herein, it does leave open avenues for further investigation. One of the most salient remaining questions is whether or not heterotopic progenitors produce neurons, and if so, do these neurons become part of the CP or heterotopia? Based on their expression of Pax6 and Tbr2, transcription factors of the glutamatergic neurogenic cascade (Hevner, 2006), it is reasonable to hypothesize that they are neurogenic. However, inasmuch as pallial radial glia are known to produce astrocytes when neurogenesis concludes (Voigt, 1989), it is possible that a subset of heterotopic progenitors might produce astrocytes, particularly at later developmental ages. Future studies may address this question in several ways. While *in utero* electroporation at E16.5 was unable to label any heterotopic progenitors, a similar approach earlier in cortical plate development at E14.5 may be able to do so. Thus, progeny of labeled progenitors could also be followed, and their fates could be established via examination of cell-type specific markers. Alternatively, if *in utero* electroporation proves ineffective for labeling heterotopic progenitors, focal electroporation using a neocortical slice culture system may be of benefit. Similarly, once the tish gene is identified, transgenic approaches could be employed to express GFP using cell-type specific promoters in developing mouse neocortex, allowing for lineage tracing experiments to be conducted.

Another important question for future study is how heterotopic progenitors disrupt neuronal migration from the normally-positioned progenitor compartments of the VZ/SVZ. The results of studies presented in this thesis indicate that at least some of the neurons that reside in the CP and heterotopia are produced by the pallial VZ/SVZ (Fig. 3-

4). Thus, the presence of abnormally-positioned proliferative cells in the IZ/CP should affect neuronal migration from the VZ/SVZ. While such an error in migration could be due to a cell-autonomous defect caused by the tish mutation, it is likely that heterotopic progenitors disrupt guidance cues in the local environment. While a complete list of important chemoattractive molecules has not been established, it is known that gradients of reelin and semaphorin 3A originating in the marginal zone and CP are important for guiding radial migrating neurons to the CP (Tissir and Goffinet, 2003; Chen et al., 2008). Moreover, a gradient of CXCL12, a chemokine produced by IPCs of the pallial SVZ and cells of the marginal zone, is critical for keeping tangentially migrating interneurons in organized streams in the SVZ and marginal zone (Tiveron et al., 2006). That IPCs have been shown to produce a molecule that is chemoattractive for neurons raises an intriguing possibility. Perhaps heterotopic progenitors can produce other chemoattractive molecules that might disrupt gradients of migratory cues for radially migrating neurons. It is known that reelin production in tish$^{-/-}$ neocortex is restricted to an appropriate location in the marginal zone (Lee et al., 1998b); however, future studies could examine other candidate chemoattractive molecules, such as CXCL12 and semaphorin 3A, to determine if disruptions in chemokine gradients might contribute to errors in radial migration in tish$^{-/-}$ neocortex. CXCL12 would be of particular interest because previous work has demonstrated that inappropriately distributed parvalbumin-positive interneurons are a rational substrate for epileptogenesis in the tish$^{-/-}$ neocortex (Trotter et al., 2006). The developmental cause for such a distribution error might be the production of heterotopic CXCL12 gradients by abnormally-positioned IPCs.

It is important to recognize that the results of the present studies cannot rule out the possibility that heterotopic progenitors of the tish$^{+/-}$ and tish$^{-/-}$ neocortex might originate from a non-pallial source. While the molecular markers expressed by heterotopic progenitors are most consistent with the interpretation that these abnormally-located progenitors originate from radial glia of the pallial VZ, this hypothesis must be verified. Initial experiments should focus on labeling pallial radial glia early in neurogenesis (E14.5) either via *in utero* electroporation or *in vitro* electroporation of slice cultures. If these experiments do not indicate a pallial source, then examination of non-pallial structures such as the ganglionic eminences, cortical hem, preoptic area, pallial-subpallial boundary, and septum, would be warranted.

## Clinical Significance

As mentioned above, there are human patients with lissencephaly and SBH for whom no causative gene or cellular mechanism has been established (Leventer, 2005; Delatycki and Leventer, 2009). While it is possible that some of these cases might be explained by as-yet undiscovered genes that are critical for neuronal migration, it is important to consider other possible causative mechanisms for this spectrum of disorders. Therefore, a broader goal of research employing the tish rat is to elucidate causative mechanisms for SBH formation that may be beneficial in understanding human cases of classical lissencephaly whose cellular mechanisms remain elusive.

Thus far, there have been no reports of human cases of SBH that involve heterotopic progenitor cells. Realistically, the identification of such a phenotype would be challenging, as it would require post-mortem analysis of embryonic neocortex, which

would be available only in cases of spontaneous or elective abortion of affected fetuses. Rather, the causative genes identified thus far have been cloned from affected patients, and the molecular mechanisms have been analyzed using transgenic and RNAi approaches in developing rodent neocortex. The utility of such approaches, and their subsequent generalizability, is complicated by significant differences between rodent and human neocortical development. For instance, the rodent neocortex is naturally lissencephalic, lacking the gyri and sulci that characterize human neocortex. In addition, the developing human neocortex appears to contain a more diverse population of restricted and unrestricted progenitor cells (Howard et al., 2006), which likely adds to the complexity of the adult human neocortex. Disruptions in rodents that lead to primary errors in neuronal migration have been assumed to generalize to human neocortex because of the similarities in neuronal migration between the two organisms. However, in light of the apparent differences in progenitor diversity between rodents and humans, an important goal for future studies will be to define how cellular, molecular, and genetic changes in animal models relate to those observed in humans. The fact that neurogenic radial glia and restricted neuronal precursors have been identified in humans is reassuring in this regard (Howard et al., 2006), as these cell types are similar in their output to radial glia and IPCs in the rodent neocortex. Ultimately, identification of the tish gene in rat neocortex will be required before sequencing studies in humans could be undertaken to determine whether the human homolog of the tish gene is involved with human cases of neocortical malformation.

## Concluding Remarks

The results presented in these studies demonstrate that mislocalized progenitor cells in the developing neocortex represent a novel mechanism underlying SBH formation. Whether or not the tish mutant will ultimately prove useful for understanding human cases of SBH for which no causative mechanism has been identified, studies utilizing this animal model have and will continue to provide important information about neocortical development and epileptogenesis. These benefits on their own make the tish rat a beneficial model for study. However, the potential for translational benefit for patients suffering from intractable epilepsy secondary to neocortical malformations makes this animal an especially attractive model for continued research.

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