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A Role for Cas Adaptor Proteins During the Establishment of the Cortical Glial Scaffold

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1- Cortical development overview

1-1 Cytoarchitecture of mammalian neocortex

The cerebral cortex, the largest and most elaborate part of the mammalian brain, is outermost layer of the brain responsible for higher cognitive function such as perception, cognition, and motor control (Carlén, 2017; Molnár, 2004; Van Essen et al., 2018). The mammalian neocortex consists of six distinct layers based on the types and number of cells (McConnell, 1989; Molnár et al., 2019; Rakic, 1972a; Shipp, 2007). Layer I is the most superficial layer and contain few neuronal cell bodies; consisting mainly of axons, dendrites and synapses (Miller & Cohen, 2001), while layer II comprises extra granular layer and pyramidal neurons. Layer III consists of principal excitatory neurons of the cortex. They receive input from other cortical and subcortical areas and send outputs to other cortical layers. Layer IV is characterized by densely packed granule cells and receives sensory information from the thalamus, particularly well-developed in sensory areas of the cortex. Layer V, responsible for motor control and generation of voluntary movements, contains large pyramidal cells that project to subcortical structure, such as thalamus, brainstem and spinal cord. Layer VI, the deepest layer contains a mixture of cell types, including pyramidal cells, fusiform cells, and polymorphic cells. This layer sends feedback projections to the thalamus and plays a critical role in modulating sensory processing. The arrangement and connectivity of these cortical layers play a crucial role in information processing within the cortex (Shipp, 2007). Different cortical areas exhibit variation in cytoarchitecture, reflecting their specialized functions and connections(Bernard et al., 2012). The development of variations in cytoarchitecture across different cortical areas is

a complex and dynamic process involving cell proliferation (Dehay & Kennedy, 2007), migration (Nadarajah & Parnavelas, 2002), axon guidance (Hakanen et al., 2019; Kalil et al., 2011; Pearlman & Sheppard, 1996) neuronal differentiation(Bernard et al., 2012; Colantuoni et al., 2011), and experience-dependent plasticity (Kalil et al., 2011). These processes work together to shape the distinct structural and functional characteristics of each cortical area, allowing for the diverse functions and computations performed by the cerebral cortex (Jiang & Nardelli, 2016).

In mice, neurogenesis in cortex occurs within a week, specifically between embryonic day 12 and term, which is a gestation period of 19 days (Chen et al., 2017). Prior to the onset of neurogenesis, the common progenitor cells divide symmetrically and produce two additional progenitor cells during each cell division, consequently double the number of progenitor cells and increase exponentially the size of the ventricular zone (McConnell, 1995; Pontious et al., 2007). After E12 in mice, certain progenitor cells start producing neurons that move away from the ventricular zone through stem cell division. Through this asymmetrical division one postmitotic daughter cell and another dividing cell are generated (Delaunay et al., 2017). The postmitotic cell, which will develop into a neuron, detaches from the ventricular surfaces and migrates towards the pial surface, ultimately settling in the cortical plate. The other daughter cell remains attached to the cerebral ventricle surface and continues to generate unequal pairs of cells.

Several hypotheses have been proposed to understand the development and organization of the cerebral cortex, the most widely accepted model for cortical migration and lamination is radial Unit Hypothesis, proposed by Pasko Rakic in 1970s, suggests that

vertical migration of oriented cohort neurons, which generated at the same site in the proliferative ventricular zone (VZ) forms the radial structure of the cerebral cortex (Rakic, 2009). Later studies, built on radial unit hypothesis, proposed radial glial model by emphasizing the role of radial glial cells not only as guides for neuronal migration but also as neural stem cells. This model proposes that radial glial cells function as multipotent progenitor cells that give rise to both neurons and glial cells during cortical development (Noctor et al., 2001).

1-2 Migration in cortical development

One of the unique characteristics of the mammalian cortex is that all its constituent neurons are generated in the proliferative zones such as ventricular zone (VZ) and subventricular zone (SVZ) (Rakic, 2009). Cortical development follows an inside first-outside last pattern of layer formation. The earliest-generated neurons form the deep layers of the cortex, while later-generated neurons migrate past the earlier ones to form the upper layers. Neural migration is vital for establishing the laminar organization of the cortex and ensuring that specific neuronal populations are positioned properly (Marín & Rubenstein, 2003). Neuronal migration is defined as the movement of neurons from their site of origin to their destination within the developing cortex (Cooper, 2013). Two primary modes of neuronal migration involved in the developing cortex, radial and tangential migration (Hatanaka et al., 2016; Marín et al., 2010).

Radial migration occurs through the movement of young neurons along radial glial cell filaments (RGC). Newborn pyramidal neurons need to travel from their place of origin where are ventricular zone and subventricular zone to the cortical plate (CP). They migrate

along radial glial cell filaments (RGC) which serve as a scaffold extending from the ventricular zone (inner layer of the developing cortex) to the pial surface (outer layer of the developing cortex). Neurons use these radial glial fibers as a railway to migrate from the ventricular zone to their appropriate cortical layer.

As the name implies, radial glial cells are characterized by a radial shape, stretching from apical side to the pial surface. They also have molecular and cellular features of astroglia such as expressing the intermediate filaments glial fibrillary acidic protein (GFAP), the astrocyte-specific glutamate transporter (GLAST), S100β, glutamine synthase (GS) and viGFP(Götz & Barde, 2005. Previous studies have shown that the glial characteristics of radial glial cells arise at E12, and by E13 to E14, radial glial cells are accounted for most progenitor cells at the different mouse brain regions. Their presence lasts until the end of neurogenesis and neural migration when they become astrocyte progenitors (Alvarez-Buylla et al., 2001; Mori et al., 2005).

This type of migration usually happens during the movement of neurons parallel to the cortical surface and perpendicular to the radial glial fibers(Barber & Pierani, 2016). Neurons undergoing tangential migration often migrate over long distance, contributing to the lateral expansion of the cortex. Tangential migration facilitates the process of populating different cortical regions with specific neuronal subtypes and contributes to the establishment of functional connectivity. In the cortex, the main population of cells that migrates tangentially are GABAergic inhibitory neurons. Birth dating studies have shown that these GABA-releasing interneurons are generated in the ventricular zone of the subpallium and then migrate tangentially across long distances to reach the appropriate

cortical layers. Once they reach their destination, they integrate into the cortical circuitry and help establish the balance between excitation and inhibition, crucial for proper brain function (Marín & Rubenstein, 2001).

1-3 Adhesion signaling pathways involved in cortical development

Neuronal migration is a complex and highly coordinated process that involves a wide array of molecular and cellular mechanisms. The precise spatiotemporal regulation of these signaling pathways is crucial for the proper development and functioning of the cerebral cortex (Jiang & Nardelli, 2016). Two canonical steps of the cell migration are formation and disassembly of adhesions between different cells, or between cells and extracellular matrix molecules (ECM). Cells can adhere to each other or to the extracellular matrix through transmembrane glycoproteins called cell adhesion molecules or CAM(Chaffey, 2003). There are four leading CAM groups in the brain including cadherin, integrins, selectins and immunoglobin superfamily. While integrins are a major class of CAMs that are highly concentrated at focal adhesions (FA), other CAMs can also be associated with these structures, particularly in the context of cell-to-cell interactions. Focal adhesions are dynamic multiprotein complexes which play as contact points for the cell with others cell or environmental cues, and facilitate the communication of the cell with surrounding extracellular environment. They act as mechanical connections between the actin cytoskeleton inside the cell and the ECM outside the cell (Parsons et al., 2010). Focal adhesions evolve from nascent cell-ECM adhesions (Wu, 2007), which in turn result from interactions of cell surface receptors such as integrins with adhesive ECM proteins. Focal adhesions can become more stable fibrillar adhesion, or depending on the nature of

extracellular stimuli, can be dissembled (Geiger et al., 2001; Murphy-Ullrich, 2001). While cells move across a surface, focal adhesions (FAs) engage with the extracellular matrix (ECM) to create the needed forces for propelling the cell's body ahead. Afterward, the cells need to detach from the ECM to sustain their movement. This underlines that effective cell migration in a specific direction relies on the ongoing, synchronized establishment and replacement of FAs at the forefront of the cell and the subsequent detachment of these connections at the rear (Nagano et al., 2012).

1-3-1 Integrin signaling pathways

Integrins are a family of cell surface receptors that play a crucial role in cell adhesion and cell signaling through crosstalk with extracellular matrix (ECM), and adjacent cells as well(Hood & Cheresh, 2002; Stachowicz, 2023). They are heterodimeric transmembrane proteins composed of two subunits, α (alpha) and β (beta), combination of 16 different α and eight different β forms various integrin receptors in different tissues (Miller & Cohen, 2001). Integrins are involved in multiple cellular events through mechanical and biochemical signaling, including cell adhesion, cell migration, signal transduction, cytoskeletal organization and development and tissue morphogenesis (Venstrom & Reichardt, 1993). They interact with specific ligands, such as fibronectin, collagen, laminin, and various cell adhesion molecules, enabling cells to adhere to their surrounding environment. Integrins are also bidirectional transmembrane signaling receptors, transmitting signals in two ways: inside-out manner, and outside-in manner (Qin et al., 2004). Outside-in signaling occurs when integrins bind to extracellular ligands, initiating intracellular signaling pathways that regulate cell behavior. Inside-out signaling

refers to the activation of integrins from inside the cell, which modulates their affinity for ligands and affects cell adhesion and migration. Furthermore, integrins play a crucial role in cytoskeletal reorganization by linking the ECM to the cell's cytoskeleton, transmitting mechanical forces, maintaining cell shape, and controlling cytoskeletal dynamics. In the cortex, the integrin signaling pathway components are involved in various processes as well, including neuronal migration, axon guidance, synaptogenesis, and synaptic plasticity (Miller & Cohen, 2001). Integrin receptors are present on the surface of neurons and interact with extracellular matrix proteins, such as laminin and fibronectin, as well as cell adhesion molecules, such as NCAM (neural cell adhesion molecule) and cadherins (Meighan & Schwarzbauer, 2008). The specific integrin heterodimers expressed in the cortex may vary depending on the developmental stage and cell type(Armendáriz et al., 2014; Miller & Cohen, 2001). Focal adhesion kinase (FAK) is a non-receptor protein tyrosine kinase that is a central component of integrin signaling (Schlaepfer et al., 1999; Zachary, 1997). It is activated upon integrin engagement and plays a critical role in neuronal migration and axon guidance in the developing cortex(Navarro & Rico, 2014). FAK phosphorylation leads to the recruitment of signaling molecules and the activation of downstream pathways. Src family kinases, including Src and Fyn, can be activated downstream of integrin engagement and contribute to FAK phosphorylation and subsequent signaling events(Schlaepfer et al., 1999). Src family kinases are critical for neuronal migration, axon growth, and synapse formation in the developing cortex. Integrin signaling in the cortex involves activation of PI3K, which is recruited to focal adhesions upon integrin engagement(Wang et al., 2015).

1-4 Malformations of cortical development

Malformations of cortical development (MCD) results from a failure of the normal process of cortical development and associated with a wide array of neurological disorders such as epilepsy and developmental delays (Pang et al., 2008). The etiology of MCD can be varied from genetic mutation to environmental factors occurring at different time period of cortical development, and at different stages of cortical development including neuronal proliferation, neuronal migration, and neuronal organization. Various types of the cortical development malformations are explained below:

1-4-1 Malformations related to abnormal cell proliferation

Microencephaly: Microencephaly or small brain characterized by an abnormally small head size and underdevelopment of the brain. It can result from abnormal cell proliferation or cell apoptosis (Passemard et al., 2013). Causes of microcephaly can vary and may include the genetic predispositions or environmental factors such as prenatal infections, exposure to certain drugs or toxins during pregnancy such as seizure, intellectual disability, and developmental delays (Gilmore & Walsh, 2013).

Hemimegalencephaly: Hemimegalencephaly is a rare neurological condition characterized by abnormal enlargement or overgrowth of one hemisphere (half) of the brain(Flores-Sarnat, 2002). Hemimegalencephaly often presents with a variety of neurological symptoms, including seizures, developmental delays, intellectual disability, motor impairments, and sometimes visual or sensory abnormalities. Hemimegalencephaly is often sporadic. However, it can also be associated with genetic mutations or genetic syndromes.

Focal cortical dysplasia: Focal cortical dysplasia (FCD) is a complex and diverse condition characterized by abnormal development of specific areas (focal regions) within the cerebral cortex (Kabat & Król, 2012). A variety of adverse events during pregnancy such as exposure to toxins or infections and hypoxia can contribute to FCD.

1-4-2 Malformations related to abnormal cell migration

Abnormal cell migration in the cortex can lead to various cortical malformations. These malformations are typically the result of disrupted processes during brain development, specifically during the migration of neurons from their place of origin to their final destinations in the cerebral cortex.

Periventricular heterotopia (PH): Periventricular nodular heterotopia is a condition characterized by clusters or nodules of neurons that fail to migrate away from the lateral ventricular zone. This condition is likely a result of a problem in the initial stage of migration in a specific group of neurons, while most neurons migrate successfully to the cerebral cortex (Lu & Sheen, 2005). Pathologically, the nodules are composed of neurons and glial cells that appear normal, along with myelinated fibers and gliosis. While some individuals with PH can be asymptomatic, the majority of them may develop seizure throughout their lives in particular during adolescence (Lian & Sheen, 2015). Periventricular heterotopia (PH) can occur due to genetic mutations or external causes. Mutations in the FLNA and ARFGEF2 genes are associated with PH. FLNA and ARFGEF2 play roles in cell adhesion and vesicle transport. Disruption of neuroependymal integrity, vesicle trafficking proteins like αSnap, and actin regulation may contribute to heterotopia formation. In mouse models, the loss of MEKK4 and αSnap function also

results in heterotopia formation, suggesting the involvement of MAP kinase signaling, vesicle trafficking proteins, and actin regulation (Parrini et al., 2006).

Subcortical band heterotopia: In subcortical band heterotopia, brain MRI reveals two distinct layers of gray matter: a thin outer ribbon and a thick inner band. These layers are separated by a thin layer of white matter. Individuals with subcortical band heterotopia may develop seizure and developmental delay that their severity directly are correlated to the degree of migration arrest (Momen & Momen, 2015). Mutations in the microtubule-associated DCX gene are responsible for causing subcortical band heterotopia. The DCX protein plays a crucial role in directing neuronal migration by controlling the organization and stability of microtubules, which are essential for neuronal movement. This malformation is observed exclusively in females since the *DCX* gene is located on the X-chromosome. Due to X- inactivation, only some of the neurons lose doublecortin function. These neurons are unable to migrate properly into the cortex, leading to the formation of the subcortical band heterotopia. In contrast, neurons that express the normal *dcx* gene migrate successfully into the cortical plate. In males with *dcx* mutations, classical lissencephaly, characterized by a smooth brain surface, is typically observed.

Classical (Type I) Lissencephaly: Lissencephaly or "smooth brain" is characterized by lack of gyri or sulci in brain. The severity of lissencephaly can vary, ranging from a complete absence of gyri (agyria) to a partially formed gyral pattern (pachygyria) or the presence of subcortical band heterotopia with some normal gyri (Verloes et al., 2007). In lissencephaly, the cortical layers are not properly formed, typically consisting of only 4 layers instead of the normal 6 layers. The specific symptoms

and severity of autosomal recessive lissencephaly can vary among individuals. Typically, affected individuals experience severe intellectual disability, developmental delays, epilepsy, and motor impairments. Several genes are involved in key processes during brain development, particularly neuronal migration, which is crucial for the proper organization and formation of the cerebral cortex. Mutations in these genes can disrupt these processes, leading to the characteristic features and symptoms of classical lissencephaly. One of the best characterized disease-causing genes is *Lissencephaly 1* (*LIS1*). Mutations in the *LIS1* gene are responsible for autosomal dominant forms of lissencephaly (Dobyns et al., 1993). *LIS1* is involved in neuronal migration and plays a crucial role in the development of the cerebral cortex. Mutations in the *dcx* gene, doublecortin, X-linked dominant are also associated with X-linked dominant lissencephaly. In males mutation in DCX, the same gene that is associated with X-linked dominant heteropia in females, causes lissencephaly in a recessive manner. *Dcx* is important for neuronal migration and the proper formation of the cerebral cortex. *Tuba1a*, Tubulin alpha 1A, which encodes for a protein called alpha tubulin, is involved in the structure and function of microtubules important for neuronal migration. Mutations in the *tuba1a* gene result in autosomal dominant lissencephaly. *Aristaless* is another gene involved in brain development and regulating neuronal migration. Mutation in this transcription factor can cause X- linked dominant lissencephaly. Mutations in the *RELN* gene can give rise to a form of lissencephaly known as autosomal recessive lissencephaly. *RELN* encodes for a protein called Reelin, which plays a critical role in neuronal migration and lamination within the cerebral cortex. In individuals with mutations in the *RELN* gene, the production or function of the Reelin protein is affected. This

disruption impairs the normal guidance and migration of neurons during brain development, leading to the malformation of the cerebral cortex seen in lissencephaly. The absence or deficiency of Reelin in autosomal recessive lissencephaly results in disrupted lamination of the cortex (Folsom & Fatemi, 2013). Instead of the normal six-layered structure, the cortex may exhibit fewer distinct layers or show disorganization. This abnormal cortical development is a hallmark of lissencephaly (Friocourt et al., 2011). Understanding the role of the *RELN* gene and the Reelin protein in neuronal migration has provided valuable insights into the pathogenesis of lissencephaly.

1-4-3 Disorders due to abnormal neuronal migration arrest

Cobblestone (Type II) lissencephaly: Cobblestone lissencephaly, also called type II lissencephaly, is characterized by the presence of nodules in the cerebral cortex due to the disrupted organization of the cortical layers. This condition is caused by the abnormal migration of neurons, which extend beyond the brain's pial surface and into the leptomeninges (Niewmierzycka et al., 2005). Cobblestone lissencephaly is often associated with eye abnormalities and congenital muscular dystrophies. Cobblestone lissencephaly is classified into three different classes based on its severity. The mild form is associated with Fukuyama congenital muscular dystrophy (FCMD); infants with FCMD typically exhibit hypotonia and generalized weakness (Niewmierzycka et al., 2005). The moderate form of cobblestone lissencephaly is seen in muscle-eye-brain disease (MEB) (Santavuori et al., 1989). In MEB, individuals experience early-onset severe myopia, glaucoma, optic disc pallor, and retinal hypoplasia as characteristic eye findings. Mental retardation, and congenital muscular dystrophy are also observed in patients with MEB. The most severe

form of cobblestone lissencephaly is associated with Walker-Warburg Syndrome (WWS), which typically leads to death within a few months after birth (Vajsar & Schachter, 2006). Cobblestone lissencephaly is inherited in an autosomal recessive manner. Four genes have been identified to be associated with this condition. WWS is linked to mutations in the *POMT1* and *POMT2* genes, Finnish MEB disorder is associated with mutations in the *POMGnT1* gene, and FCMD is caused by mutations in the Fukutin gene. All of these genes play a role in the glycosylation of a protein called alpha dystroglycan. Alpha dystroglycan is a highly glycosylated protein that acts as a receptor for various molecules in the extracellular matrix, maintaining the stability of the cell surface (Niewmierzycka et al., 2005). Mutations in the identified genes disrupt the glycosylation process of alpha dystroglycan, compromising the integrity of the extracellular matrix adhesion complex that led to a weakening of the structural integrity of the superficial marginal zone or the cortex (Nickolls & Bönnemann, 2018). As a result, migratory neurons can "over-migrate" beyond this structural barrier and reach the pial surface, causing the characteristic cobblestone or bumpy surface appearance. Hartmann et al. showed that disruption of the basal lamina along the brain surface also leads to a cobblestone phenotype in mice (Hartmann et al., 1998). They found out deficiency in the transmembrane protein Presenilin1 (PS1), known for its involvement in the processing of amyloid precursor protein, can result in a cortical dysplasia resembling cobblestone lissencephaly (Herms et al., 2004). PS1 deficiency affects the support provided by leptomeningeal cells to Cajal-Retzius cells and the maintenance of the pial basement membrane. This disruption leads to both the over-migration of neurons and the premature termination of neuronal migration. Pathological

examinations reveal the loss of Cajal-Retzius neurons in the marginal zone, depletion of the extracellular matrix protein reelin, and chondroitin sulfate (Wines-Samuelson et al., 2005).

1-5 CAS proteins and cell migration

CAS family (CRK-associated substrate) is involved in focal adhesion turnover. P130Cas or simply CAS, a 130 KD protein, contains a Src-homology 3 (SH3) domain at the amino terminal followed by two proline-rich regions and a substrate domain consisting of fifteen 15 repeats of a four amino acid sequence (tyrosine-any two aminoacids-proline;YXXP) (Huang et al., 2007a; Tang, 2009). The YXXP motif found in the substrate-binding domain can serve as SH2-binding ligand following phosphorylation (Tikhmyanova et al., 2010). Other two members of Cas family share homologous structure and sequence with p130Cas: human enhancer filamentationn HEF1 (CasL) and Embryonal Fyn- associated substrate (Efs)/Src- interacting protein (Sin). These share a similar domain structure with Cas, with the highest sequence similarity in the SH3 domain and the carboxy terminal 200 amino acids. One of the most significant differences between the functional sequence of these proteins is the absence of a proline- rich motif in HEF1. This proline-rich region binds to the Src SH3 domain and pTyr-containing sequences that function as binding domains to the Src SH2 domain in p130Cas and Efs/Sin.

1-5-1 Functional role of Cas proteins during cortical development

The mechanism of cell migration is closely connected to interactions between cells and the extracellular matrix (ECM). The first indication that Cas played a role in this process originated from studies that detected Cas proteins in focal adhesions, which are

sub-cellular structures forming molecular links between the ECM and the actin cytoskeleton (Bouton et al., 2001). As discussed before, integrins as receptors for extracellular matrix can activate several pathways involving in cell migration. Phosphorylated Cas proteins play a role in one of these pathways as an adaptor proteins. They are engaged in the recruitment of Crk, activation of Rac, and actin cytoskeleton remodeling (Bouton et al., 2001; Defilippi et al., 2006). Moreover, *in vitro* studies have shown that Cas signaling adaptor proteins mediate integrin signal transduction during neural development (Bargon et al., 2005).

Three *Cas* genes are expressed in the developing CNS including the dorsal telencephalon (Merrill et al., 2004). The dorsal telencephalon is divided into the cerebral cortex, which gives rise mainly to the neocortex and the hippocampus; and the dorsal midline, which gives rise to the choroid plexus and the cortical hem (Campbell, 2003). Our lab showed that *p130Cas, CasL,* and *Sin* are expressed in proliferative zones in wild type (WT) mice in embryonic stage E12.5 and E14.5 using RNAscope assay (Wong et al., 2022). Overlap of expression in the proliferative zone during development indicates their possible functions during cortical development including neurogenesis, migration, and lamination.

To test for a requirement for *Cas* genes during cortical development, we performed genetic ablation of *p130Cas*, *CasL* and *Sin*. Considering this fact that the loss of *p130Cas* causes embryonic lethality (Honda et al., 1998), and the overlapping expression and the redundant roles of these genes, we generated the triple knock out mouse model to meet the experiments needs. A cortical-specific *p130Cas* ablation was generated in a $CasL^{-/-}$;$Sin^{-/-}$ double null mutant genetic background and referred it to the triple conditional knock-outs:

"TcKO") (Donlin et al., 2005). In $Sin^{-/-}$, the first exon, the first intron, and part of the second exon was removed leading to the disruption of SH3 domain. In $CasL^{-/-}$, the exon coding SH3 domain was replaced by EGFP and neomycin resistance gene (Neo) cassette through homologous recombination in ES cells (Seo et al., 2005). To drive cre recombinase expression, we used the *Emx1cre* line. *Emx1Cre* can be detected in the medial and dorsal and lateral pallia and is expressed in both progenitor cells and post-mitotic neurons and restricted only to projection neurons (Gorski et al., 2002). The absence of *Cas* family expression was confirmed through on E14.5 in *Emx1Cre;CasTcKO* (Wong et al., 2023)

Examination of cortical structure in *Emx1Cre;CasTcKO* using pan-neural markers suggest a significantly different cortical phenotype from the control. In *Emx1Cre;CasTcKO*, the cortical surface is bumpy and displays cobblestone features. Nissl staining revealed the presence of empty pocket in the cortical plate. Moreover, displacement of cortical neurons was observed using mature neuronal marker NeuN (Wong et al., 2022). As we know, each layer of 6 layered cortical structure is characterized with a population of neuron with specific cell morphology, connectivity, or expression of specific transcription factors (Carlén, 2017). In this prior study, examined the cortical laminar structure in *Emx1Cre;CasTcKO* animals, through immunohistochemistry (IHC) and using markers for transcription factors that maintain their cell lineage identity (Wong et al., 2022). Wong et al. reported the abnormal positioning of both deep and superficial layer neurons in *Emx1Cre;CasTcKO* at P7 compared to the control. CTIP2+ and TBR1+ cell clusters were detected in the upper cortical plate and Cux1+ neurons in the lower cortical plate. RoR-ß+ cells in layer IV showed a wave- like appearance invading the superficial cortical

plates and meninges. These results suggest that Cas adaptor proteins are required for proper cortical lamination

After these initial studies, a few questions remained unanswered. First, it is not clear if the misplacement of layer specific markers in *Emx1Cre;CasTcKO* animals resulted from defects in migration and neural positioning, or defects in neuronal fate specification. Second, the onset of the cortical developmental defects was not established. In this study, we explored the underlying reasons for cortical layer misplacement. Plus, we investigated the onset of the (abnormal phenotype) in *Emx1Cre;CasTcKO* mice.

1- Methods and Materials:

2-1 Animals and genotyping

All animal procedures were performed according to the University of California, Riverside Intuitional Animal Care and Use Committee (IACUC. The day of vaginal plug observation was assigned as embryonic day of 0.5 (E0.5) and the day of birth was considered as postnatal day 0 (P0).

Generation of Triple knock out mice

Since CasL and Sin compensate for the lack of p130Cas, we performed specific p130Cas ablation in a CasL-/-; Sin -/- double null mutant genetic background.

Emx1-Cre (B6.129S2-Emx1tm1(cre)Krj/J) was purchased from the Jackson Laboratory (Strain no. 005682). Genotyping for *Emx1-Cre* was performed using the following primers: Forward primer: CCA TAT CAA CCG GTG GCG CAT C and Reverse primer: TCG ATA AGC TTG GAT CCG GAC AG. Tail biopsy samples were digested and stabilized using Quanta tail extraction reagent kit (Quantabio, 95091-025) and PCR reactions prepared using GoTaq Master Mix (Promega, PRM7123). In the next step, PCR reaction was performed using PCR BIORad C1000-Touch Thermal cycler and following program: 95˚C - 5 min, 95˚C – 1min, 61˚C - 45 sec, 72˚C - 30 sec, 35X, 72˚C - 10 min,4˚C.

2-2 Immunohistochemistry

Embryonic brains were dissected in cold 1× Phosphate-buffered saline (PBS) and postnatal mice were perfused with cold 4% paraformaldehyde (PFA) in 1× PBS (ThermoFisherScientific,28906), then dissected. The brain samples were post fixed in 4% PFA for two hours, four hours and overnight at 4˚C followed by washing with 1× PBS next

day. The samples were sunk in 30% sucrose (ThermoFisherScientific, J65148.A1) overnight at 4°C prior to the cryopreservation. The dehydrated samples were embedded in a mold using Embedding Medium Tissue-Tek OCT Compounding Clear (Sakura, 4583) and stored at -80°C. In the next step, the cryopreserved brain samples were sectioned coronally at 20 µm on cryostat (Leica, CM3050S).

Cryosection samples were blocked for one hour at room temperature using blocking buffer: 0.1% TritonX-100 (ThermoFisherScientific, BP-151100), 10% Goat serum and 1× PBS in a humidifier chamber prior to primary antibody addition. Primary antibodies including rabbit anti cux1, 1: 250 (Santa Cruz, sc-514008) , rabbit anti- CTIP2 , 1:500 (Abcam, 18465), rabbit anti TBR1, 1:500 (Abcam, 31940) , anti laminin, 1:500 (Abcam, ab11575), anti Nestin (Abcam, ab134017) and anti rabbit TBR2 (Abcam, ab23345) were diluted in antibody dilution buffer 0.1% Triton-X100, PBS and 5% goat serum. The samples were incubated in the primary antibody mix overnight at 4°C; followed by washing with PBS for 15 minutes x 8 in a slide mailer. In the next step, the cryosection samples were incubated with the proper secondary antibody for the primary antibody of interest at 1:500 concentration including: goat anti-rat 488 (ThermoFisherScientific, A-21208), goat anti-rat 546 (ThermoFisherScientific, A-11081), goat anti-rabbit 546 (ThermoFisherScientific, A-11035), goat anti-chicken 488 (ThermoFisherScientific, A-110309), goat anti-chicken 647 (ThermoFisherScientific A-21449). Additionally, to visualize DNA, DAPI was used at 1 µg/ml (ThermoFisherScientific, 50850585) in the antibody dilution buffer. The cryosection slides were incubated with secondary antibody mix overnight at 4°C, followed by washing at room temperature with the PBS 15 minutes

x 4 times in a slide mailer, and slides were mounted using Fluorogel with DABCO™, Anti-Fading Mounting Medium (Electron Microscopy Sciences, 17985-10).

2-3 EdU Cell Proliferation Assay

5-ethynyl-2'-deoxyuridine, EdU is a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis.

EdU (ThermoFisherScientific, 10044) was dissolved in DMSO (ThermoFisherScientific, 295522500) and diluted with sterile water to 15 mg/ml at the time of injection. EdU was injected into the intraperitoneal cavity of 150 µg/kg at E12.5 and E15.5 and collected after 30 min or 7 days. The brain samples were dissected and cryopreserved according to the instruction explained for the immunofluorescence.

I performed Edu proliferation assay using *Click-iT® EdU Alexa Fluor® 488 Imaging Kit* (Thermo Fisher Scientific, C10632). The kit contains all components needed to label DNA-synthesizing cells and to detect EdU incorporated into DNA. Initially, the cryosection samples were blocked with 3% bovine serum albumin (BSA) in 1× PBS at pH 7.4 for one hour at room temperature; followed by permeabilization step using 0.5% triton-X100 in 1× PBS. The samples were washed with 3% BSA in × PBS for 5 minutes.

To label EdU incorporated DNA, the cryosection samples were stained using Alexa Fluor™ 488, Click-iT® EdU reaction buffer and $CuSO_4$, Alexa fluor azide 488 and reaction buffer additive (1X).

The cryosections were co labeled with Cux1 for E15.5 animals collected at P3 and TBR1 for E12.5 collected at P0.

2-4 Confocal imaging

The confocal scanning images were acquired Leica DMi8 using a 5x, 10x and 20x objective lenses. The confocal microscope was equipped with four laser lines for excitation of fluorophores. The laser lines and their corresponding wavelength were as follows: 405 nm (blue) for DAPI excitation, 488 nm for FITC excitation, 567 nm for RFP excitation, 640 nm for Alexa fluor 647 excitations.

The emission filers used to collect fluorescence signals were selected based on the specific fluorophores employed in the study: DAPI (blue) emission was collected through a 420-480 nm filter, FITC (green) emission was collected through a 500-550 nm filter, RFP emission was collected through a 570-620 nm filter, Alexa Fluor 647 (red) emission was collected through a 660-710 nm filter.

The pinhole size was set to 1 Airy unit to ensure optimal confocality, reducing out-of-focus light and increasing image resolution. The system allowed for Z-stack imaging to capture multiple focal planes through the sample, facilitating 3D reconstructions and analysis.

2-5 Quantification of layer Markers/Edu and Statistical analysis

Raw images of cryosection samples were converted to TIFF files using Fiji and quantified using the RapID cell counter, an open-source program developed by Sekar and collogues (Sekar et al., 2021). The thickness of the cortex was divided into 10 equal bins, bin 1 corresponds to the marginal zone (MZ) and bin 10 corresponds to ventral layer. RapID automatically detected and quantified fluorescent cell bodies. The analysis parameters were set according to the fluorophore of interest, maximum sigma, minimum

sigma, threshold and overlap. The proportion of the cells in each bin= number of cells labeled in bin divided by total number of labeled cells. The graph values are mean ± standard error of mean, n=3-5 independent samples per group, and 3 sections per samples. The dataset was tested for normality using the Shapiro-Will test and QQ plot. The Mann whithney *U* test was performed Bonferroni correction or Benjamin-Hochberg with false discovery rate<0.1 and $p<0.05$ (Benjamini et al, 2001).

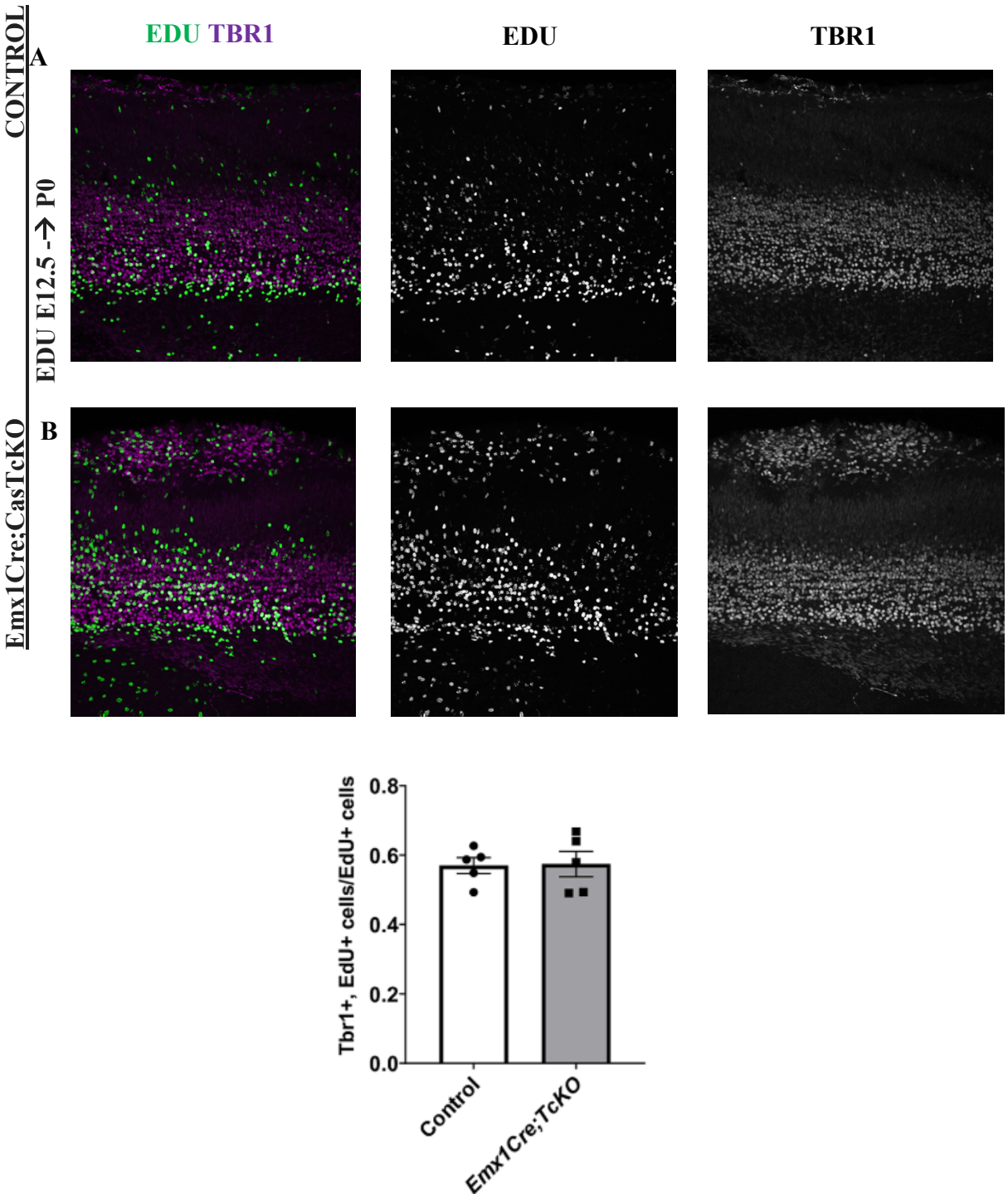
2- Results

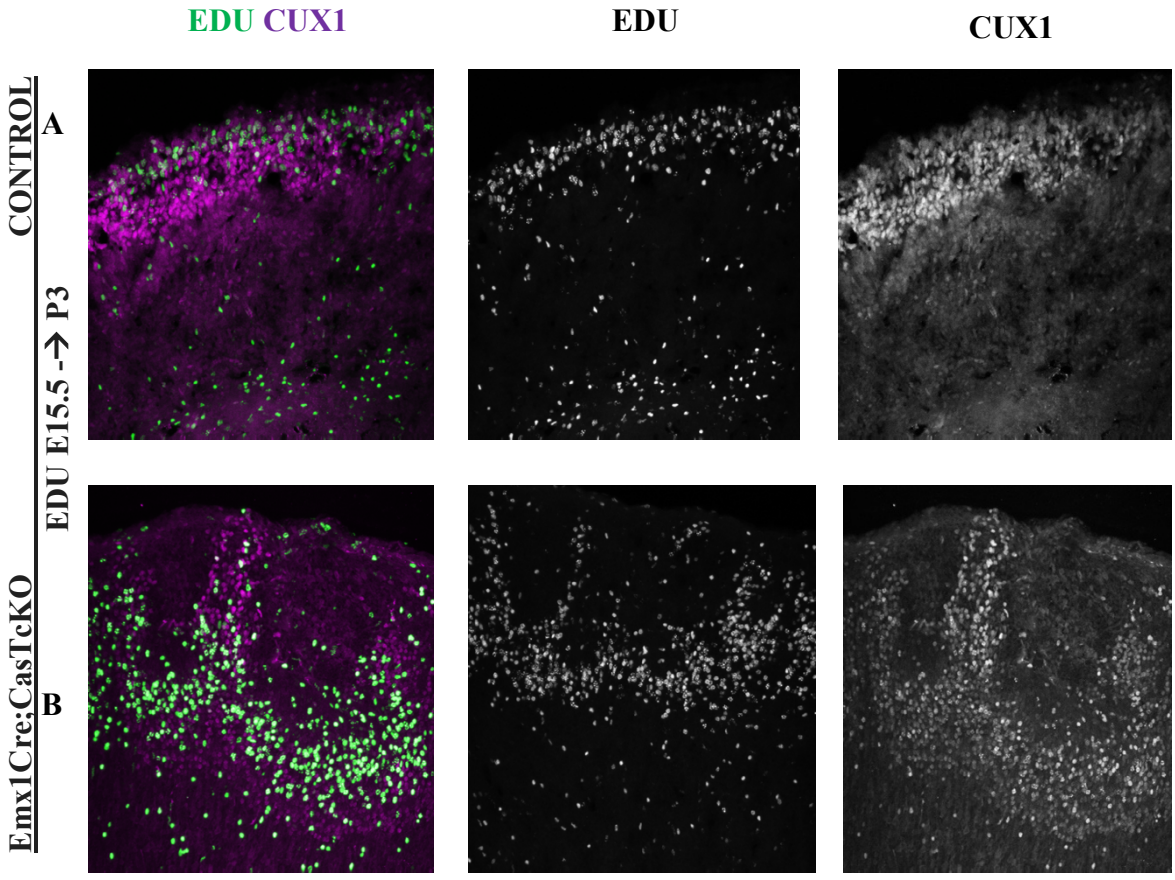
3-1 Cortical dysplasia in is due to a neuronal mispositioning defects

To distinguish if the misplacement of layer-specific markers observed in *Emx1Cre;CasTcKO* animals result from defects in migration and neuronal positioning or defects in neural specification, Wong et al treated newly born cells during the course of radial migration by performing Ethynyl deoxyuridine (Edu) pulse chase experiments. Edu, a nucleoside analog of thymidine, is incorporated in cells undergoing the S-phase of the cell cycle and can be used to observe the final position of cells born at a specific timepoint. Edu was administered to pregnant dams at E12.5 or E15.5 to label newly born deep and superficial layer neurons, respectively. Collection at seven days post-intraperitoneal injection for each timepoint (E12.5->P0) and (E15.5->P3) is sufficient for these populations of excitatory neurons to complete radial migration. Scattered columns of Edu+ cells locate closer in the pial surface (Bin1) in *Emx1Cre;CasTcKO* animals compared to the controls, while, in the control, they tend to settle in the layer VI. More remarkable differences are observed when animals are pulsed at E15.5 and collected at P3. Prospective superficial neurons that are Edu+ are spread across the ten bins in *Emx1Cre;CasTcKO* animals, whereas in the control mice these Edu+ cells are primarily positioned closer to the pial surface. This misplacement of cells appears to be caused, in part, by columns of ectopic Edu cells in the cortical plate. These data suggest that the cortical dysplasia observed in *Emx1Cre;CasTcKO* animals is likely due to a neuronal mispositioning defects.

To further confirm that the lamination defects are caused by a migration defect, I repeated the pulse chase experiments and co-labeled the Edu+ neurons with layer specific

markers. It is expected that if the cells born at a particular timepoint are misplaced without changing their fate, they should maintain their expression of the appropriate cortical layer marker. In control animals 57.0 ± 8% neurons labeled by Edu at E12.5 also co-express the deep layer marker Tbr1 at P0. A comparable proportion of the Edu+ neurons was co-labeled by EdU and Tbr1 in P0 *Emx1Cre;CasTcKO* animals that were treated in the same way **(Figure 1-A).** When animals were pulsed with EdU at E15.5 and the brains were collected at P3 to label superficial layer neurons, the percentage of EdU+ neurons that co-expressed the superficial layer marker Cux1 was also very similar between control and *Emx1Cre;CasTcKO* **(Figure 1-B)**. These results confirm that EdU+ cells in *Emx1Cre;CasTcKO* mice are properly specified, and thus are most likely mispositioned due to migration defect.


CONTROL
A
EDU E12.5 -→ P0
EDU TBR1
EDU
TBR1
Emx1Cre;CasTcKO
B
Tbr1+, EdU+ cells/EdU+ cells
0.8
0.6
0.4
0.2
0.0
Control
Emx1Cre;TcKO


EDU CUX1
EDU
CUX1
CONTROL
A
EDU E15.5 -→ P3
Emx1Cre;CasTcKO
B

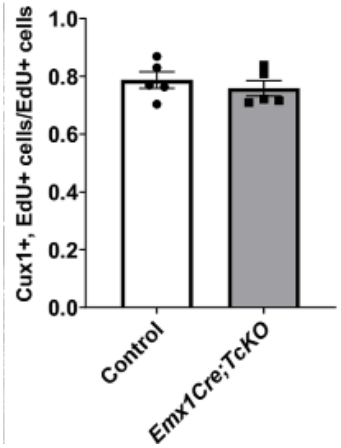

Cux1+, EdU+ cells/EdU+ cells
1.0
0.8
0.6
0.4
0.2
0.0
Control
Emx1Cre;TcKO

Figure 1- Genetic ablation of *Cas* genes in cortical progenitors results in misplacement of neurons

(**A-B**) Detection of prospective deep layer (**A**) and superficial layer neurons (**B**) labeled by EdU (green) at E12.5 and E15.5, respectively, on coronal sections of control and *Emx1Cre;CasTcKO* cortices. Sections were stained for deep layer marker Tbr1 (**A**), or superficial layer marker Cux1 (**B**) shown in magenta. Scale bars for lower magnification panels in A,B: 75 μm. CP, cortical plate; EdU, ethynyl deoxyuridine; IZ, intermediate zone; MZ, marginal zone; SVZ, subventricular zone; VZ, ventricular zone.

3-2 Onset of cortical lamination defects occurs between E12.5 and E15.5

When is the cortical dysplasia phenotype first observed in the *Emx1Cre;CasTcKO* mutants By P0, *Emx1Cre;*CasTcKO animals already display an overt disruption of cortical organization (**Fig 1A; Wong et al. 2022**), suggesting a possible embryonic onset of this phenotype (Wong et al., 2022). We investigated the onset of the abnormal phenotype in *Emx1Cre;CasTcKO* mice. To visualize the dysplasia and ectopias at embryonic stages, we labeled E12.5 and E15.5 control and *Emx1Cre;CasTcKO* cortices for Tbr1, a marker that labels the preplate (PP) or majority of the cortical plate (CP) at those stages, respectively **(Figure 2- A-D)**. In E12.5 *Emx1Cre;CasTcKO* cortices, the localization of Tbr1+ neurons appeared similar to the controls **(Figure2-A)**. However, by E15.5, ectopic neurons breaching the pial surface were clearly visible in *Emx1Cre;CasTcKO* embryos, a phenomenon not observed in control animals (**Figure2-B,C)**. By this stage, *Emx1Cre;CasTcKO* animals already show ectopic Tbr1+ and Ctip2+ cells positioned outside of the compromised basal lamina (stained with Laminin) **(Figure2-D)**. On average, at this stage, we observed 2.84 ± 0.3 ectopias/mm of cortical surface length in *Emx1Cre;CasTcKO* embryos but observed 0 in controls **(Figure2-E)**; Mann–Whitney *U* test, $*p < 0.05$, two-tailed test, $n = 4$ animals per genotype). These results establish the developmental onset of the cobblestone phenotype in *Emx1Cre;CasTcKO* cortices between embryonic days 12.5 and 15.5.

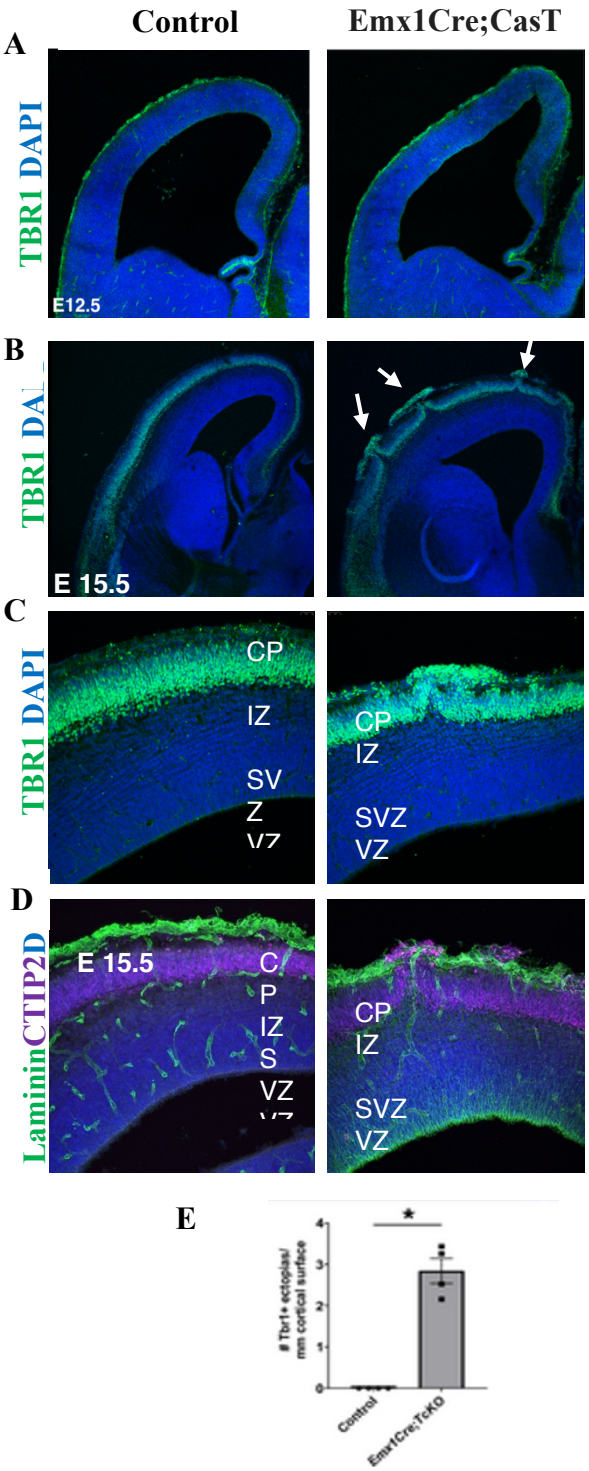

Control
Emx1Cre;CasT
A
TBR1 DAPI
E12.5
B
E 15.5
C
CP
IZ
SV
Z
VZ
CP
IZ
SVZ
VZ
D
Laminin CTIP2D
E 15.5
CP
IZ
SVZ
VZ
CP
IZ
SVZ
VZ
E
Tbr1+ ectopias/ mm cortical surface
Control
Emx1Cre;TcKO

Figure 2- Developmental onset of cortical dysplasia in pancortical *Cas* mutants.
(A-E) Coronal sections of control and *Emx1Cre;CasTcKO* cortices at E12.5 (A), and E15.5 (C-D) stained for Tbr1 (green) (A, B, C), or Ctip2 (magenta) and Laminin (green) (D). While there are no obvious differences observed between *Emx1Cre;CasTcKO* and controls at E12.5 (B), by E15.5, there are clear ectopias breaching through the pial surface of *Emx1Cre;CasTcKO* cortices (C-D). (E) Quantification of the number of ectopias per length of cortical surface in mm (E). Values provided are mean ± SEM. *$p < 0.05$, Mann–Whitney *U* two-tailed test, $n = 4$ animals per genotype, 3–5 sections per animal. For data plotted in graphs. Scale bars: A: 250 μm; B: 500 μm; C and D: 75 μm. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone.

3-3 Programmed cell death is not responsible for cortical dysplasia phenotypes in *Emx1Cre;CasTcKO* mice

The cobblestone phenotype could be partially caused or compounded by changes in programmed cell death during early developmental time points. We examined the levels of cleaved (active) Caspase-3 in control and *Emx1Cre;CasTcKO* cortices at different developmental stages to visualize the levels of apoptosis before (E12.5), right after (E15.5), and several days after (P0 and P3) the onset of the cortical dysplasia phenotype **(Figure 3 A-D)**. The density of caspase-3 activation in control and *Emx1Cre;CasTcKO* mice was not significantly different at any of these stages (Mann–Whitney *U* two-tailed test, $p \geq 0.7$ for all stages, $n = 3$ animals per genotype). These data suggest that programmed cell death is not severely disrupted in *Emx1Cre;CasTcKO* cortices and is unlikely to be a primary cause of the ectopias and cortical dysplasia phenotypes observed in these mice.

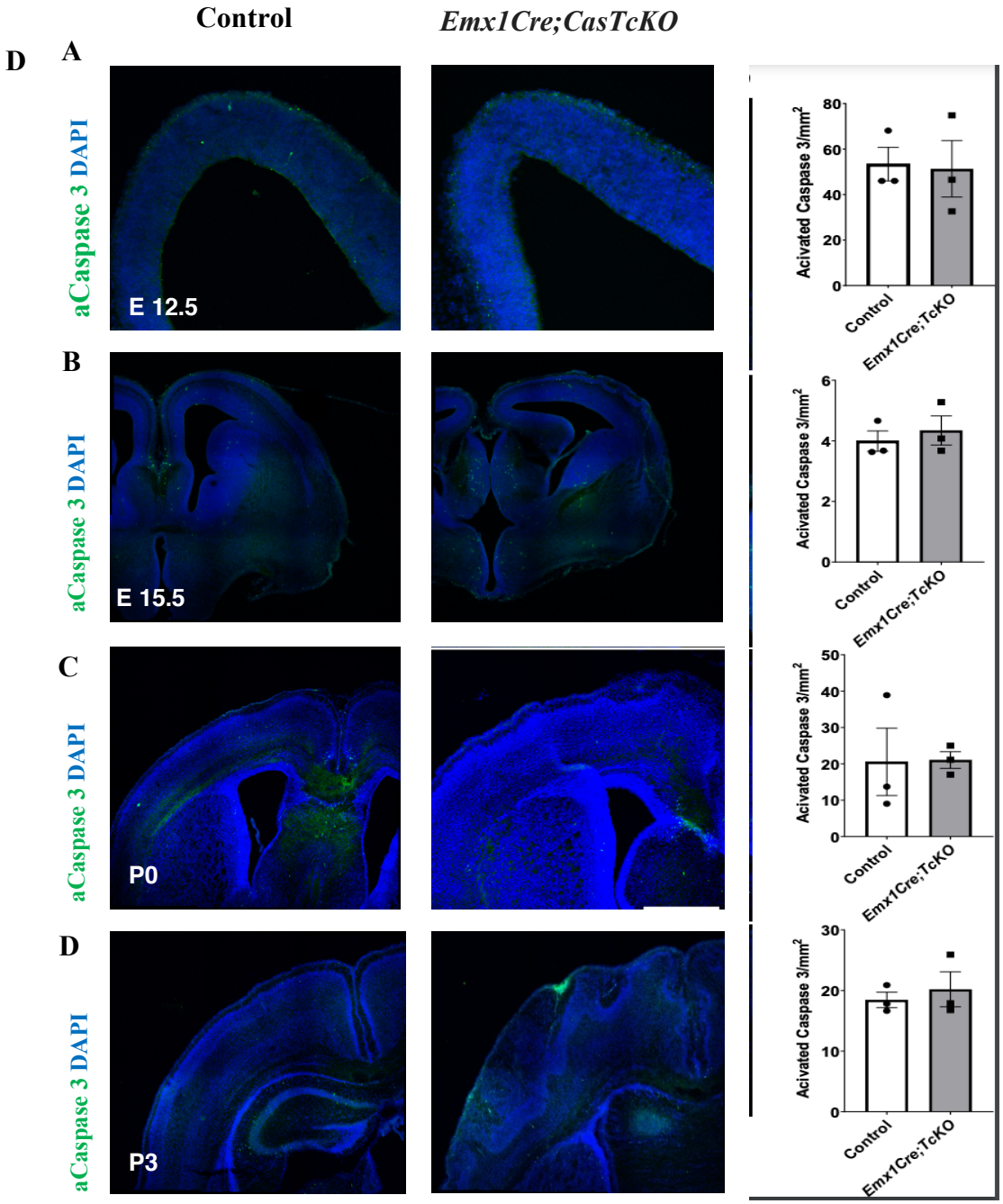

D
Control
Emx1Cre;CasTcKO
A
aCaspase 3 DAPI
E 12.5
B
aCaspase 3 DAPI
E 15.5
C
aCaspase 3 DAPI
P0
D
aCaspase 3 DAPI
P3
Acivated Caspase 3/mm2
Control
Emx1Cre;TcKO

Figure 3- Caspase3 activation is not affected in *Emx1Cre;CasTcKO* mice at key developmental stages.

(A-D) Immunostaining for activated Caspase3 (aCasp3, green) on E12.5 (A), E15.5 (B), P0 (C), or P3 (D) coronal sections of control and *Emx1Cre;CasTcKO* cortices. Sections were counterstained with DAPI (blue). No notable differences are observed between genotypes. Right panels: quantification of density of activated Caspase3. Values given are mean ± SEM, $n = 3$ independent samples per group, 3–5 sections per sample, Mann–Whitney *U* test and Bonferroni correction, ns for all stages ($p \geq 0.7$). Scale bars for A: 100 μm; B, C, D: 500 μm**.**

3-4 Cas function is required for the maintenance of the embryonic pial base membrane and radial glial cell interaction

The development of the cortex relies on the vital interactions between the radial glial endfeet and the embryonic pial basement membrane. According to previous work from our lab, the absence of the typical "cobblestone appearance" and the presence of an intact basement membrane in *NexCre;CasTcKO* cortices suggest that *Cas* genes are acting in a non-neuronal autonomous manner during cortical lamination. These also imply that the disruptions in cortical layers observed in *Emx1Cre;CasTcKO* cortices may stem from malfunctioning RGCs.

To test whether *Cas* genes are required for the embryonic pial basement membrane and radial glial endfeet integrity, we performed histological assessment of the cortical scaffold of *Emx1Cre;CasTcKO* at E15.5 **(Figure 4-A)**. While no obvious defects were observed in control animals, there are several disruptions to the glial–pial interface in *Emx1Cre;CasTcKO* cortices. There is widespread rupture of the basal lamina as indicated by regions with breached Laminin staining. The Nestin+ RGCs do not make proper adhesion contact with the basal lamina at the exposed Laminin sites where basal processes extend into the subarachnoid space. These results suggest that *Cas* function is required for the maintenance of the embryonic pial basement membrane–RGC interactions.

To determine whether the disrupted basement membrane–RGC interaction caused mispositioning of proliferating progenitor cells as observed in other models of cortical heterotopia and dysplasia(Bargon et al., 2005; Kielar et al., 2014), EdU was again administered to *Emx1Cre;CasTcKO* animals (**Figure4-B and Figure 5**). However,

collection was 0.5 hours following injection at E12.5 **(Figure-5)** or E15.5 (**Figure4-B**) for these experiments. This short EdU pulse labels the proliferating cells during neurogenesis of deep layer and superficial layer neurons, respectively. We then stained these brains for EdU and the intermediate progenitor marker Tbr2. At E12.5, EdU+ proliferating neural progenitors are positioned in the SVZ and VZ **(Figure5-A**). At this stage, a few proliferative cells are also observed in the PP of both controls and *Emx1Cre;CasTcKO* animals **(Figure5)**. At E15.5, proliferative cells are mainly restricted to the SVZ and VZ of control animals with a few cells labeled by EdU in the CP and MZ. In *Emx1Cre;CasTcKO* animals, EdU+ cells are also primarily observed in the SVZ and VZ (**Figure 4-B)**. While occasionally a few EdU+ cells were localized to the MZ and upper CP regions, no significant differences were observed between the density of proliferative cells in the CP of *Emx1Cre;CasTcKO* and control animals (**Figure 4-C)**. This indicates that the breakage in the basement membrane and disruption of radial glial endfeet observed in *Emx1Cre;CasTcKO* cortices does not result in ectopic positioning of progenitor cells. To test whether the overall levels of proliferation and neurogenesis were affected, we measured the density of EdU+ cells in E12.5 and E15.5 control and *Emx1Cre;CasTcKO* cortices (**Figure4-D and Figure5-C**). Importantly, the density of proliferating cells is not significantly different between these mutants and controls (Mann–Whitney *U* two-tailed test, $p = 0.34$ for E12.5 and $p = 0.89$ for E15.5, $n = 4$ animals per genotype). To further examine possible defects in neurogenesis and to test whether the transition from RGC to intermediate progenitor is disrupted in *Emx1Cre;CasTcKO* cortices, we quantified the proportion of EdU+ cells that

coexpresses Tbr2. This proportion is not significantly different between control and *Emx1Cre;CasTcKO* animals at E12.5 **(Figure 5-B,D)** or E15.5 **(Figure 4-B and E);** Mann–Whitney *U* two-tailed test, $p = 0.49$ for both stages, $n = 4$ animals per genotype), suggesting that the disruptions in cortical lamination observed in these mutants are also unlikely to be caused by an early transition from RGC to intermediate progenitor fate.

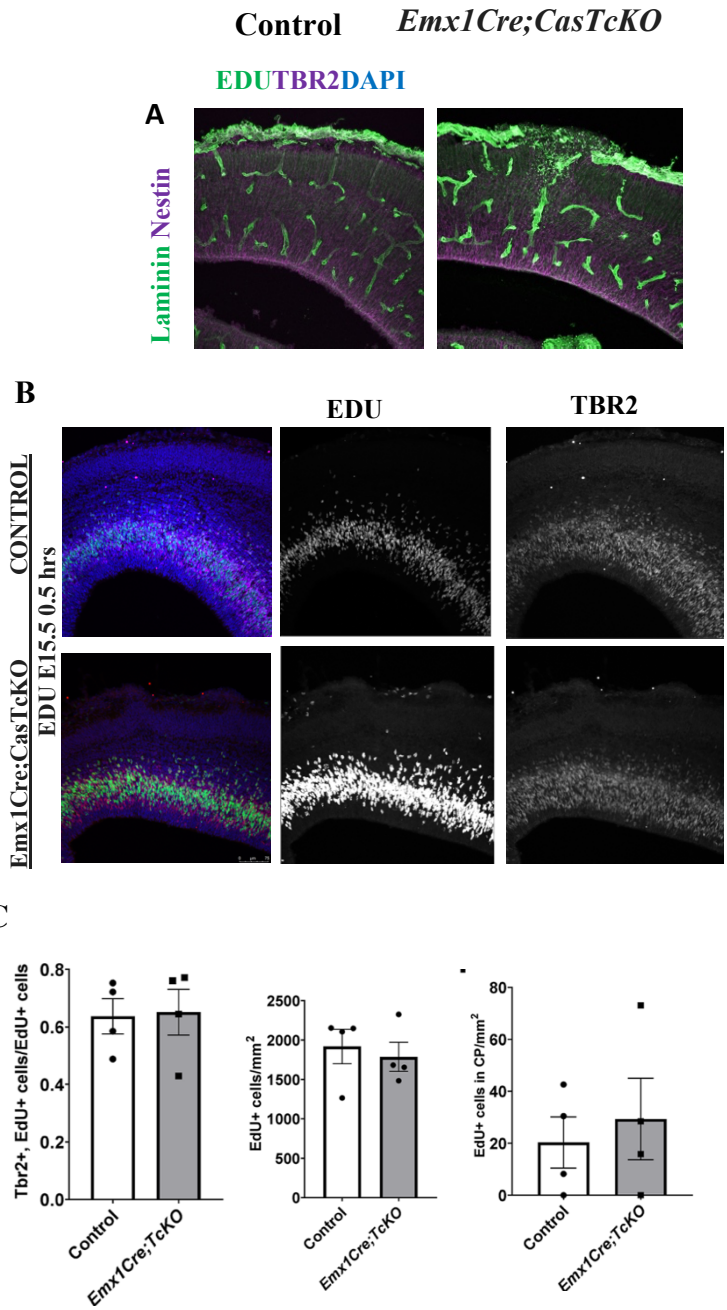

Control
Emx1Cre;CasTcKO
EDUTBR2DAPI
A
Laminin Nestin
B
EDU
TBR2
CONTROL
Emx1Cre;CasTcKO
EDU E15.5 0.5 hrs
C
Tbr2+, EdU+ cells/EdU+ cells
0.8
0.6
0.4
0.2
0.0
Control
Emx1Cre;TcKO
EdU+ cells/mm2
2500
2000
1500
1000
500
0
Control
Emx1Cre;TcKO
EdU+ cells in CP/mm2
80
60
40
20
0
Control
Emx1Cre;TcKO

Figure 4- Cas proteins are critical during radial glial scaffold formation
Laminin (green) and Nestin (magenta) antibody staining on E15.5 control and *Emx1Cre;CasTcKO* coronal sections. Radial glial endfeet do not make proper contact with the basal lamina in *Emx1Cre;CasTcKO* animals at exposed Laminin sites (arrows). (B) Detection of proliferative cells using EdU (green) at E15.5, colabeled with Tbr2 (magenta). Proliferating cells are occasionally observed within the CP of control and *Emx1Cre;CasTcKO* animals (white arrows). (C) Quantification of density of EdU+ cells in CP. Mann–Whitney *U* two-tailed test, $p = 1$, $n = 4$, 4 sections per animal. (D) Density of EdU+ cells per area of cortex. Mann–Whitney *U* two-tailed test, $p = 0.89$, $n = 4$ animals per genotype, 3 sections per animal. (E) Proportion of EdU+ cells that coexpress Tbr2. Mann–Whitney *U* two-tailed test, $p = 0.49$, $n = 4$ animals per genotype, 3–5 sections per animal. Values given are mean ± SEM. Scale bars: 100 μm. CP, cortical plate; EdU, ethynyl deoxyuridine; MZ, marginal zone; VZ, ventricular zone

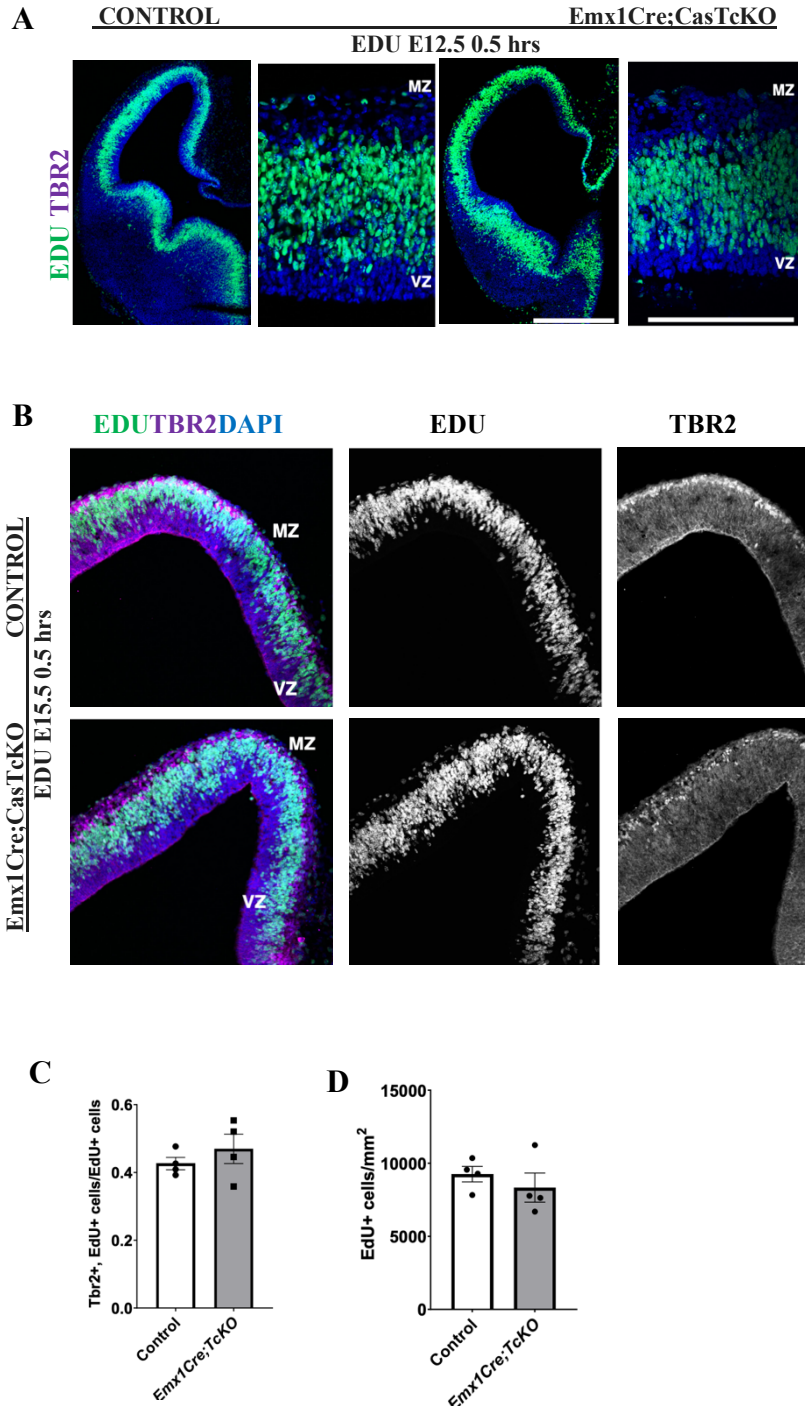

A
CONTROL
Emx1Cre;CasTcKO
EDU E12.5 0.5 hrs
EDU TBR2
MZ
VZ
B
EDUTBR2DAPI
EDU
TBR2
CONTROL
EDU E15.5 0.5 hrs
Emx1Cre;CasTcKO
C
Tbr2+, EdU+ cells/EdU+ cells
0.6
0.4
0.2
0.0
Control
Emx1Cre;TcKO
D
EdU+ cells/mm2
15000
10000
5000
0
Control
Emx1Cre;TcKO

Figure 5- Progenitor phenotype in E12.5 *Emx1Cre;CasTcKO* cortices.
(A, B) Detection of proliferative cells in control and *Emx1Cre;CasTcKO* coronal sections using short-pulsed EdU at E12.5 (A, B, green), colabeled with the intermediate progenitor marker Tbr2 (B, magenta). Nuclei were counterstained with DAPI (blue). (C) Density of EdU+ cells/mm^2 of cortex. $p = 0.34$, two-tailed Mann–Whitney *U* test, $n = 4$ independent samples per group, 3 sections per sample. (D) Proportion of Edu+ cells that coexpress Tbr2. $p = 0.49$, two-tailed Mann–Whitney *U* test, $n = 4$ independent samples per group, 2–3 sections per sample. Values given are mean ± SEM. Scale bars: Lower magnification panel for A: 500 μm; Higher mag panels in A: 100 μm; B: 75 μm.

3- Discussion

During early CNS development, lamination and stratification serve as intricate blueprints, directing the systematic positioning of neuronal populations into discrete layers or strata, each endowed with distinct characteristics, connectivity patterns, and functional roles (Carlén, 2017; Shipp, 2007). Radial glial cells serve as a critical scaffold in the intricate process of neuronal migration during the development of the nervous system (Marín et al., 2010; Rakic, 1972a). In this study, we shed light on a molecular mechanism that underlies the assembly of the glial scaffold, facilitating the intricate processes of cortical migration and lamination. Our findings unveil an adhesion signaling pathway which operates within radial glial cells (RGCs) to uphold the interactions between glial endfeet and the pial basement membrane.

Glial scaffold establishment and maintenance is critical during cortical lamination. The accurate attachment and interaction between radial glial endfeet and pial basement membrane are crucial for establishment of the glial scaffold (Hatanaka et al., 2016; Rakic, 1972b). It has been shown that disruption in these events can lead to of significant defects in cortical layering, such as cortical dysplasia and a cobblestone cortex phenotype (Halfter et al., 2002; Hu et al., 2007). The cortex undergoes substantial expansion throughout neurogenesis. As new layers emerge and become integrated, RGC processes must adapt and extend to accommodate this rapid cortical expansion to maintain spatial confinement of neurons and preventing aberrant migration of cell bodies (Halfter et al., 2002). Active adherence of RGC endfeet to the basement membrane is vital for maintaining scaffold structure. Hence, the dynamic regulation of adhesion signaling, and cytoskeletal

remodeling plays a pivotal role in facilitating these dynamic interactions between glial cells and the pial membrane.

Previous research in our lab showed that three *Cas* family genes are expressed in cortical plate and proliferative regions during embryonic development in mice (Wong et al., 2022). Moreover, the investigation of cortical phenotype in *Emx1Cre;CasTcKO* using pan neural markers and cortical layer markers showed abnormal positioning of both deep and superficial layer neurons which leads to the cobblestone phenotype, whereas the control mice exhibit the smooth cortical surface. We demonstrated that the onset of disrupted cortex exhibiting comprised lamina and misplaced $TBR1^+$ and $CTIP2^+$ cells occur between the embryonic day of 12.5 and 15.5. In the next step, pulse chase experiments and co-labeling Edu^+ cells with cortical layer markers ruled out that the cell fate specification errors caused the misplacement of cortical layers. These findings are consistent with recent studies showing that the importance of Cas adaptor proteins for cell migration and axon pathfinding (Huang et al., 2007b; Riccomagno et al., 2014; Vahedi-Hunter et al., 2018). Moreover, another underlying reason for cobblestone phenotype can be modifications in programmed cell death(Bizzotto & Francis, 2015). Our results demonstrated that the expression of activated caspase- 3, a marker for apoptosis is similar between *Emx1Cre;CasTcKO* and controls and the cobblestone phenotype is not due to the increase of the programmed cell death.

One of the main functions of the radial glial cells is providing a scaffold for neurons(Marín et al., 2010; Nadarajah & Parnavelas, 2002). Interaction between embryonic pial basement and radial glial endfeet are essential for the cortical development.

According to studies from our lab, ablation of *Cas* genes in only post-mitotic neurons using *NexCre* suggests non-neuronal autonomous role of Cas in cortical migration: Cortical phenotype characterization of *NexCre;CasTcKO* showed no difference at cell positioning in cortex compared to the controls. Taking this into account, we tested the role of *Cas* genes in interaction of the embryonic pial basement membrane and radial glial endfeet(Wong et al., 2023). Our results suggest that Nestin+ RGC do not establish a proper adhesion contact with the basal lamina in *Emx1Cre;CasTcKO* at E 15.5 . In addition to this, short EdU pulse labeling experiments in *Emx1Cre;CasTcKO* at E 15.5 demonstrates that cortical lamination defects in these mutant are not due to the early transition of the radial glial cells to the intermediate progenitor fate.

Although the work described in this thesis makes it possible to understand molecular pathways better, further investigation is still needed for comprehending how Cas protein function is regulated. Dystroglycan (Dag1) is a ubiquitous ECM receptor that could potentially be acting upstream of Cas adaptors. Dag1 glycosylated extracellular subunit directly interacts with ECM componenets, while its intracellular subunit interacts with cytosolic proteins, mostly involving in cytoskeletal remodeling(Nickolls & Bönnemann, 2018). Myshrall et al previously demonstrated that dystroglycan mediated interactions between pial base membrane and radial glial cells is necessary for pial base membrane integrity (Myshrall et al., 2012). Moreover, Satz et al showed that *NestinCre dag1*$^{flox/flox}$ showed the phenotype of breach of the pial basement membrane which resembled the cortical phenotype of the *Emx1Cre;CasTcKO*(Satz et al., 2010). Considering these similarities, further studies on dysroglycan from our lab led by Wong et al showed that

Emx1Cre *Emx1Cre;Dag1*$^{flox/-}$ phenocopies *Nestin Cre,* *Dag*$^{flox/flox}$ in cortical development(Wong et al., 2023). In addition to this, Dag1 can augment the p130Cas phosphorylation *in vitro*. To determine whether Dag1 can signal directly to regulate Cas phosphorylation and function during cortical lamination, they examined the cortical lamination phenotype of a knock-in mouse which only expresses the extracellular domain of Dag1. Somewhat surprisingly, they found no evidence of ectopias or cortical dysplasia in these mutants compared to controls. Thus, the intracellular domain of Dag1 is not necessary for cortical lamination.

Riccomagno et al already showed that Cas phosphorylation in retinal ganglion cells is dependent on ß-integrinfunction (Riccomagno et al., 2014). Histological studies by Wong et al. demonstrated that ß-integrin is necessary for cortical lamination in radial glial cell autonomous manner (Wong et al., 2023).

Taken together, the β1-Integrin may be candidate for a signal transducing receptor in radial glial cells for Dag1-dependent phosphorylation of Cas. To confirm this possibility, forced phosphorylation of pCas130 using FIT construct and histological examination of the cortex in *Emx1Cre; ß-intgr*$^{flox/flox}$ were performed and the results suggest that forced phosphorylation of p130Cas can rescue the cobblestone phenotype of *Emx1Cre;ß-intgr*$^{flox/flox}$. Therefore, we can conclude that tyrosin phosphorylation of Cas protein act downstream of β1-Integrin. These findings are aligned to with previous studies showing the conserved role of Cas proteins in neuronal migration (Wong et al., 2022). However, since phosphorylated Cas proteins have not been detected *in vivo* yet, it is still not known

whether these adhesion module can be modulated by other signaling pathways remains to be seen.

Through the examination of phenotypic characteristics, genetic rescue experiments, and molecular epistasis data, a functional model has been suggested for Dag1-Integrin-Cas adhesion axis. This model suggests this axis operates in at least two ways. First, this axis facilitates the anchoring and restructuring of radial glial cells end feet as the cortex expands, this process supports the establishment and maintenance of the glial scaffold by enabling interactions with the extracellular matrix (ECM). Additionally, Dag1 and Integrin within the RGC end feet play an internal role by organizing and stabilizing the basement membrane's integrity through interactions with ECM molecules such as Laminin. This concerted establishment and maintenance of both the glial scaffold and basement membrane are imperative for facilitating proper neuronal migration and lamination processes. (Figure-6) (Wong et al., 2023). All in all, our results and further investigations in our lab have introduced promising and unexplored pathways in the investigation of the genetic origins of debilitating neurodevelopmental disorder such as cobblestone lissencephaly and focal cortical dysplasia (FCD) (Pang et al., 2008).

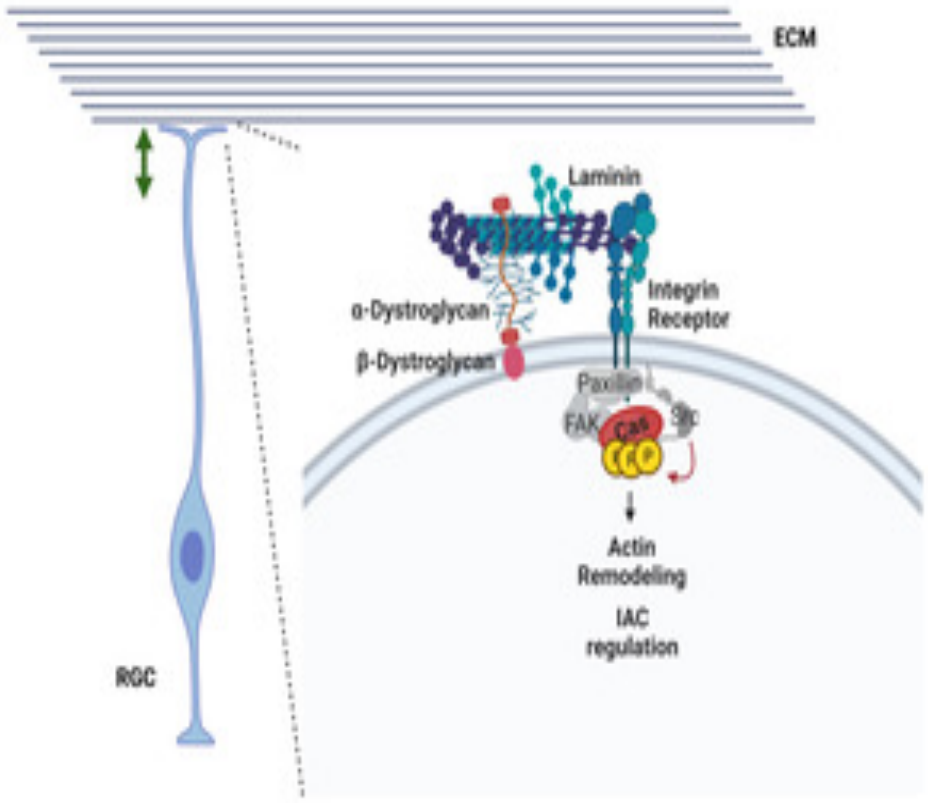


Figure 6- A working model for an adhesion signaling axis regulating radial glial cells scaffold formation and base membrane maintenance. Dag1-Integrin-Cas axis acts in cis in RGCs to maintain attachment to the basement membrane as the cortex expands, In addition, Dag1 and β1-integrin participate in the maintenance of the basement membrane by acting through their extracellular domains, Additional cytoplasmic IAC proteins that appear in grey have been shown to participate in cortical lamination based on genetic evidences, and we speculate that they could be acting the same pathway (Hu et al., 2007; Kawauchi, 2011; Sanes, 1989).

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