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Csde1, an RNA Binding Protein, Modulates Neuronal Subtype Specification

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Csde1, an RNA Binding Protein, Modulates Neuronal Subtype Specification

by

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Abstract

Neuronal diversity is the root of complex function in the cerebral cortex. During cortical development, neural stem cells give rise to neurons. Neurons are a diverse cell type and can be grouped into many subtypes, each with distinct functional identities. Neurogenesis and neuronal subtype specification are carefully regulated, both temporally and spatially, by programmed gene expression. One mechanism controlling gene expression is translational regulation, which alters levels of protein synthesis. Translational regulation orchestrated by RNA binding proteins allows dynamic alterations in protein expression. Here I determine that Cold-shock domain containing E1 (Csde1), an RNA binding protein, regulates the specification of neuronal subtypes in the cerebral cortex. In the developing murine cortex, Csde1 is highly expressed in newborn neurons of the cortical plate during development. I show that reduced Csde1 expression alters neuronal subtype in the embryonic and post-natal cortex. Csde1 reduction disrupts the distribution of neuronal subtypes within the cortical plate. Additionally, reduced Csde1 expression increases mixed neuronal subtype identity. Together, this indicates that the specification of neuronal subtypes is subject to translational regulation, and that Csde1 activity is essential for specification of neurons in the cortex.

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List of Abbreviations

Symbol/Abbreviation	Definition
4-ET (eif4enif1)	Eukaryotic translation initiation factor 4E transporter
Ascl1	Achaete-Scute Family BHLH Transcription Factor 1
ASD	Autism Spectrum Disorder
BDNF	Brain-derived neurotrophic factor
Brn1 (Pou3f3)	Pou Class 3 Homeobox 3
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
CNS	Central Nervous System
Cortex	Cerebral cortex
CP	Cortical Plate
CPN	Callosal projection neuron
Csde1	Cold Shock Domain Containing E1 (previously Unr)
CThPN	Corticothalamic projection neuron
Ctip2 (BCL11B)	B-cell lymphoma/leukemia 11B
DCC	Dosage compensation complex
DL	Deep layer
DOB	Date of Birth
E	Embryonic Day
Fezf2	FEZ Family Zinc Finger 2
FGF2	Fibroblast Growth Factor 2
Foxg1	Forkhead Box G1

G3BP	G3BP Stress Granule Assembly Factor 1
GATA-1	GATA Binding Protein 1
GFP	Green Fluorescent Protein
GN	Granular Neurons
HBSS	Hank's Balanced Salt Solution
hESC	human Embryonic stem cells
HRP	Horseradish Peroxidase
IF	Immunofluorescence
IHC	Immunohistochemistry
IP	Intermediate Progenitors
IRES	Internal ribosome entry site
ISH	*in situ* hybridization
IZ	Intermediate Zone
KD	Knockdown
KO	Knockout
lncRNA	Long non-coding RNA
Msl-2	Male sex-lethal 2
NDS	Normal Donkey Serum
Neurog2	Neurogenin-2
NPC	Neural Progenitor Cell
NSC	Neural Stem Cell
OE	Overexpression
P	Postnatal Day

Pax6	Paired box 6
PB	Processing Body
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
Pum2	Pumilio2
qRT-PCR	Quantitative reverse transcription PCR
RAC1	Ras-related C3 botulinum toxin substrate 1
RBP	RNA binding protein
RG	Radial Glia
RISC	RNA-induced silencing complex
RNP	Ribonucleoprotein
roX	*RNA on X*
Satb2	Special AT-rich sequence-binding protein 2
SCPN	Subcerebral projection neuron
SDS	Sodium dodecyl sulfate
SG	Stress Granule
shRNA	Short-hairpin RNA
Sox2	SRY-box 2
SPN	Subplate neurons
STRAP	Serine-threonine kinase receptor-associated protein
SVZ	Subventricular Zone
SXL	Sex-lethal
Tbr1	T-box Brain Transcription Factor 1

Tbr2	T-box Brain Transcription Factor 2
TBS-T	Tris Buffered Saline + Tween20
TF	Transcription factors
Tle4	Transducin-like enhancer protein 4
UL	Upper layer
Unr	Upstream of N-ras
VZ	Ventricular Zones
β-III	β-III Tubulin

Chapter 1: Introduction

1.1 Overview

The cerebral cortex (cortex) is a highly organized structure in the mammalian brain. The cortex is essential for higher order function and the integration of sensory information. The cortex has a laminar structure, with defined layers of distinct subtypes of excitatory neurons. These subtypes of excitatory neurons exhibit different properties, including morphology, projection to other brain regions, and molecular profiles (Leone et al., 2008; Martineau et al., 2018). The assignment of neuronal subtype allows integration of the cortex with other brain structures, such as the contralateral cortex, the thalamus, and the brain stem. Disruptions in the subtype identity of cortical neurons have cascading effects throughout the brain, altering neuronal projections and circuit formation which can ultimately result in neurodevelopmental disorders, such as autism spectrum disorders (ASD) (Varghese et al., 2017).

The specification of neuronal subtype identity is tightly controlled by the expression of key identity genes. Some of these genes are expressed in neural stem/progenitor cells (NPCs) to instruct the generation of specific subtypes of neurons whereas others are expressed at the post-mitotic stage to establish the identity of newborn neurons (Greig et al., 2013). Transcriptional regulation has been the focus of research in the past few decades and some core transcriptional programs that control neuronal subtype specification have been discovered (Greig et al., 2013). However, transcriptional programs alone may not fully account for the precise timing and abundance of neuronal diversity in the cortex. Indeed, recent studies reveal that NPCs and immature neurons show considerable transcriptional expression of cell-identity genes without the production of the corresponding proteins (Zahr et al., 2018).

Translational regulation is increasingly recognized as a critical regulator in cell fate decisions, regulating translation of key proteins to affect cellular change (Lennox et al., 2018; Luo et al., 2018; Pringle et al., 2019). In the cortex, it has been shown that regulation at the translational level also contribute to cell fate determination of NPCs during cortical development (Zahr et al., 2018). Despite these findings, we still know little about how specific translational mechanisms control neuronal subtype specification, and how perturbations of these RNA-based mechanisms contribute to neurodevelopmental disorders such as ASD. There are many mechanisms which control RNA metabolism in the cells, one of which is RNA binding proteins (RBPs). RNA binding proteins are critical posttranscriptional regulators of mRNA metabolism, oftentimes, binding multiple target RNAs to control their translation.

Recent work on an RNA binding protein Cold shock domain containing E1 (Csde1) show that it is strongly associated with ASD (Guo et al., 2019; Stoner et al., 2014). This association raises the possibility that Csde1 may regulate neurodevelopmental processes, and perturbation of Csde1 is sufficient to disrupt normal cortical development. Previous work on ASD shows disruption in cortical layering and neuronal subtype specification, further implicating Csde1 in neuronal subtype specification via its impact on translational programs of gene expression (Pucilowska et al., 2015; Stoner et al., 2014; Varghese et al., 2017). I hypothesize that Csde1 is a regulator of cortical development and may alter neurogenesis and neuronal maturation in the cerebral cortex. My project focuses on determining how Csde1 regulates the generation specification of cortical neurons.

1.2 Specific Aims

Aim 1: Csde1 expression is controlled in the developing cortex.

Previous work shows that Csde1 is expressed ubiquitously across body systems (Uhlén et al., 2015). However, Csde1 expression within the cerebral cortex is not characterized. Using cultured cells isolated from the embryonic murine cortex, I examined the expression of the *Csde1* gene and found it expressed in cortical neurons. Further examination of Csde1 expression with immunostaining and in situ hybridization revealed robust Csde1 expression in newborn neurons in the embryonic cortex.

Aim 2: Csde1 may promote neurogenesis and reduce NPC self-renewal.

During cortical development, stem cell proliferation is highly regulated. NPCs must control self-renewal and neurogenic differentiation, and changes in the ratio of NPCs to neurons can alter brain morphology. Using primary NPCs, I found that Csde1 knockdown reduces neurogenesis in NPC cultures.

Aim 3: Cortical neuron subtype identity is altered by Csde1 knockdown

Control of subtype specification is essential during cortical development. I demonstrate that loss of Csde1 alters subtype identity of cortical neurons. Knockdown of Csde1 reduces upper layer neurons with a corresponding increase in deep layer neuron subtypes. In the murine cortex, heterozygous knockout of Csde1 alters neuronal subtype. Heterozygous mice show an increased number of mixed identity neurons, which express deep layer and upper layer characteristics.

Significance

The aim of this project is to determine how post-transcriptional programming coordinated by Csde1 affects cortical development. Corticogenesis is complex and highly regulated developmental process. The number and diversity of neurons generated during this period is vast,

which leaves it vulnerable to perturbation. Perturbations in cortical development lead to various neurodevelopmental disorders. This ranges from severe cortical deformations and intellectual disability to mild alterations with mild cognitive and behavioural differences (Parenti et al., 2020). One such example is autism spectrum disorder (ASD), which has a high degree of variability between patients (Guo et al., 2019; Zhou et al., 2019). Particularly, ASD which has high genetic tractability, is classified by deficits in social communication in addition to restricted and repetitive behaviour (Ayhan and Konopka, 2019; Varghese et al., 2017).

Given the genetic basis of ASD, many ASD associated genes have been identified. Variants of the *Csde1* gene have previously been associated with ASD, as well as mild to severe intellectual disability (Guo et al., 2019). Understanding how disruption of *Csde1* alters the cortical development may provide information of ASD. Therefore, Csde1 provides a tool to understanding how post-transcriptional programming may alter cortical development, leading to functional impairments. Additionally, determining if and how post-transcriptional programs factor into development will allow us to further our model of corticogenesis. Together, investigating disruptions caused by ASD associated Csde1, and its role in translational regulation in the cortex will inform our understanding of neurodevelopmental disorders, such as ASD.

Chapter 2: Literature Review

2.1 Cortical Development

The cerebral cortex is a large, complex, and evolutionarily conserved part of the mammalian brain and is the outermost layer, covering the brain. The cerebral cortex is generally subdivided into four regions: the occipital, temporal, parietal, and frontal lobes. Each lobe has distinct functions, for example, the occipital lobe interprets visual stimuli, while the temporal and parietal lobes process auditory and physical stimulus. The frontal lobe allows integration of stimulus and is responsible for reasoning and problem solving. These functional differences are made possible by regional variability in the cellular composition of the cerebral cortex (Agirman et al., 2017).

The patterning of the cerebral cortex begins with the development of the neural tube. Neural stem cells begin as a layer of neuroepithelial cells, called the neural plate. The neural plate undergoes neurulation, folding into itself to create a hollow neural tube. Closure of the anterior neural tube creates a ventricle, a hollow space enclosed by neural stem cells (Figure 1A). It is this anterior end of the neural tube which becomes the telencephalon, and is the basis for cerebral cortex development (Hébert and Fishell, 2008).

The cerebral cortex develops from the dorsal telencephalon. The neuroepithelial cells as the earliest neural progenitor cells (NPCs) surrounding the ventricle provide the foundation for the expansion of the cortex (Figure 1B). These cells proliferate by symmetric divisions, generating additional NPCs required for neurogenesis. When neurogenesis begins, these NPCs convert into radial glia cells (RGs) that surround the ventricles to form the ventricular zone (VZ)

and extend the basal processes toward the pial surface of the developing cortex (Levitt and Rakic, 1980). RGs proliferate and differentiate between embryonic day (E) 11.5 and E13.5 in the mouse telencephalon, and generate intermediate progenitors (IPs), a secondary type of NPC. These proliferative RGs and IPs expand outwards from the ventricle to populate the subventricular zone (SVZ) (Sidman et al., 1959).

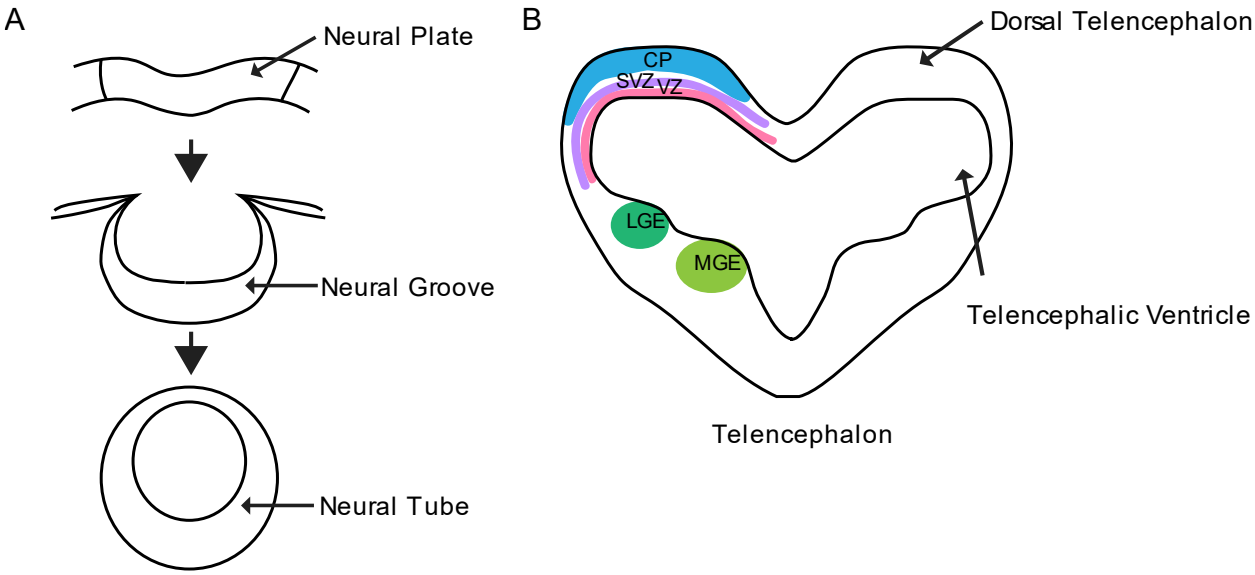

Figure 1. Neural tube formation gives rise to the telencephalon. (A) neurulation of the neural plate. The neural plate folds into itself, giving rise to a closed neural tube. (B) The telencephalon is formed at the anterior end of the neural tube. The dorsal telencephalon gives rise to cortical neurons from the ventricular and subventricular zone (VZ/SVZ, pink/purple), which migrate radially to populate the cortical plate (CP, blue). Cortical interneurons migrate tangentially from the lateral and medial ganglionic eminence (LGE, MGE respectively, green) to settle in the CP.

NPCs gradually shift from proliferative divisions to asymmetric self-renewing division. This self-renewing division maintains the NPC population, while simultaneously generating cortical neurons. Cortical neurons originate from the VZ/SVZ and migrate out into the cortical plate (CP) between E12.5-14.5 in the mouse telencephalon (Figure 2) (Angevine and Sidman, 1961; Haubensak et al., 2004). During this time, cortical interneurons generated by the lateral and medial ganglionic eminences (LGE, MGE) in the ventral telencephalon, migrate tangentially into the CP (Guo and Anton, 2014) (Figure 1B) . This interneuron migration continues throughout cortical development. During the final neurogenic stage, most NPCs switch from asymmetric self-renewing division to symmetric neurogenic division, terminally differentiating to form two neurons (Haubensak et al., 2004), while a small population of NPCs continue to generate glia cells and/or contribute to the adult neural stem cell pool. Terminal differentiation cannot occur too early, else the NPC pool will be depleted, preventing adequate neuron generation. The balance between proliferation, self-renewal and terminal differentiation is carefully controlled and relies on extrinsic signalling pathways as well as intrinsic control (Dhanesh et al., 2016; Hirabayashi et al., 2004; Hitoshi et al., 2002; Pereira et al., 2010). For example, Notch signalling in embryogenesis maintains RG identity, and loss of Notch signalling depletes the NPC pool (Dhanesh et al., 2016; Hitoshi et al., 2002; Yoon et al., 2004). Similarly, repressive histone methylation is required to maintain RG identity, and loss of this epigenetic repression shifts RGs towards differentiation and enhances neuronal gene expression (Pereira et al., 2010).

Regulation of NPC self-renewal and neurogenesis is essential, but insufficient for normal cortical development. In addition to their generation, cortical neurons generated by RGs and IPs must migrate away from the VZ/SVZ towards the cortical plate to form a stereotypical six-layer laminated structure. Each of these six-layers is enriched for different neuronal subtype. The

lamination of the cortex occurs in an “inside out” manner, with earliest born neurons occupying the deepest layers of the cortical plate and later born neurons migrating past their predecessors (Figure 2) (Angevine and Sidman, 1961; Takahashi et al., 1999).

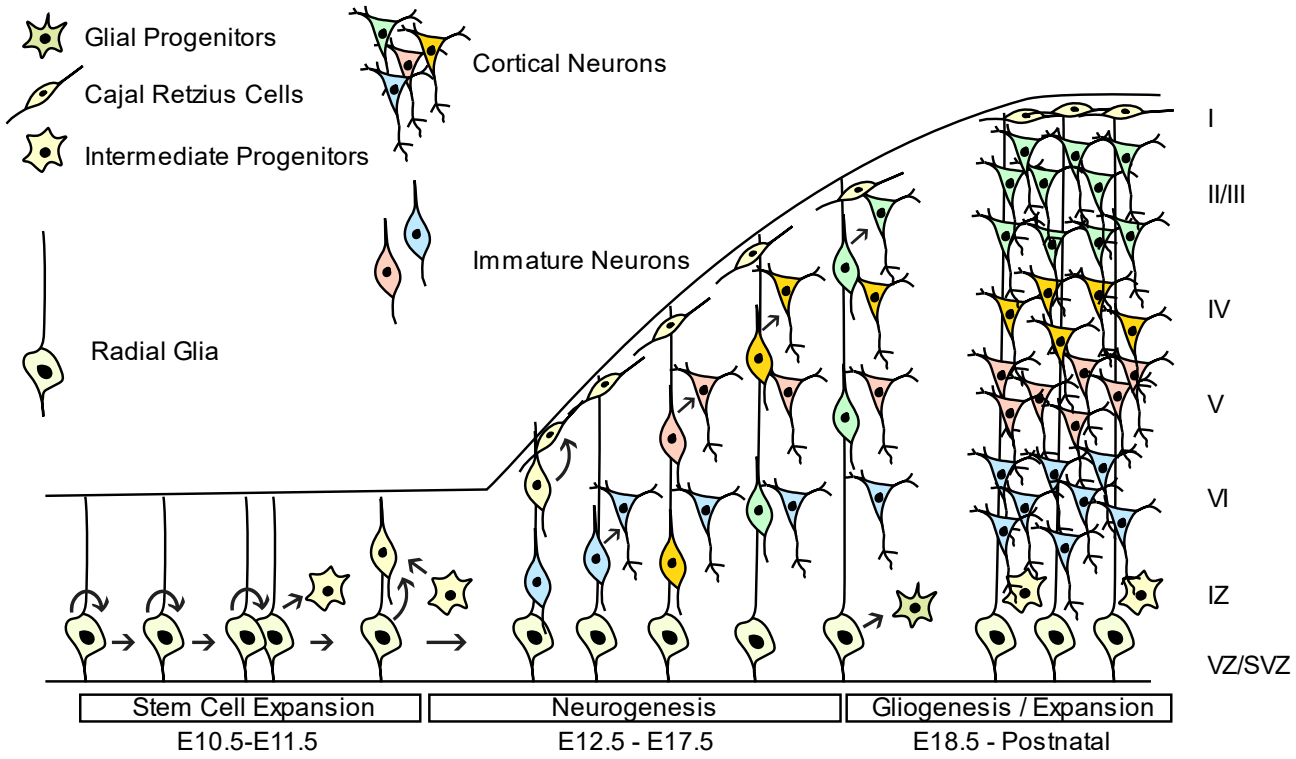

Figure 2. Schematic Diagram of Murine Corticogenesis. Neural Stem cells (ie. Radial Glia and Intermediate Progenitors) proliferate and expand the stem cell population around E10.5. Formation of the preplate occurs art E11.5, splitting to form the subplate and layer I Cajal-Retzius cells. Stem cells begin diving asymmetrically to generate neurons in an inside out fashion between E12.5 and E17.5. Early born neurons generate deep layers VI and V, and subsequent neurons migrate past the deep layers to populate the superficial layers II-IV. Gliogenesis begins around E18.5 and continues along with neurogenesis into the postnatal stages of development. Maturation and final positioning of neurons occurs in the post-natal stages.

During embryonic development, the first-born neurons in the cortex form the pre-plate, around E11.5 in the murine cortex. The pre-plate is a transient structure, which contains two subpopulations of neurons: subplate neurons, and Cajal-retzius cells. When the pre-plate is split, it forms the basal subplate and pial layer I. The area between the subplate and the pial surface is called the cortical plate (CP). Layer I Cajal-retzius cells secrete Reelin, a glycoprotein which signals subsequent neurons to migrate towards the pial surface (Frotscher, 1998). This reelin signalling is essential for the "inside-out" lamination of the cortical plate. The neurons which split the pre-plate settle between the subplate and layer I to form layer VI. Following layer VI formation, progressive waves of neurons migrate past the previous layer, guided by RG processes and environmental cues, to populate increasingly superficial layers (V-II) of the cortex (Kriegstein and Noctor, 2004; Stiles and Jernigan, 2010). Each layer of the cortex has a specific composition, and is enriched for different neuronal subtypes (Greig et al., 2013). These subtype identities are essential for lamination and circuit connectivity. Although cortical lamination is completed during the embryonic window, cortical development continues during the post-natal stage. This includes extension and refinement of neuronal projections, and continued expansion of the cortical plate.

Cortical development requires precise regulation of NPC proliferation, neurogenesis, migration, and neuronal subtype assignment. Together, these are coordinated to form a laminated, six-layer structure. Alterations in any of these developmental steps are associated with neurodevelopmental disorders, however ASD is primarily associated with disruptions in neuronal subtype specification.

2.1 Neuronal Subtype Specification

Neurons are terminally differentiated cells which arise from progenitor cells. Although neurons share common features which distinguish them as a cell type, within this broad classification neurons can adopt many different characteristics. It is the diversity in secondary characteristics that defines subtypes of neurons. For example, neurons are divided into excitatory and inhibitory subclasses, based on their signalling characteristics (Manuel et al., 2015). These neuronal subtypes are further refined using multiple criteria including molecular profile, birthdate, morphology, and projection profile (Hevner, 2007). The primary excitatory neuronal subtypes found within the cerebral cortex are pyramidal and granular neurons (Greig et al., 2013; Hodge et al., 2019).

Layer	Subtype	DOB	Function
I	Cajal-Retzius (CR)	E10.5	Aid neuron migration and lamination
II/III IV	Callosal Projection Neurons (CPN)	E14.5-16.5	Project through the corpus callosum
IV	Granular Neurons (GN)	E14.5	Project to adjacent neurons
V	Subcerebral Projection Neurons (SCPN)	E13.5	Project to brain stem and spinal cord
VI	Corticothalamic Projection Neurons (CThPN)	E12.5	Project to thalamus and surrounding cortices

Table 1. Summary of neuronal subtypes within the cerebral cortex. Simplified table of subtypes found in each cortical layer of the cerebral cortex. Date of birth (DOB) shown in embryonic days (E).

Within the cerebral cortex, these excitatory subtypes roughly segregate within the layers of the cortical plate (Table 1) (Leone et al., 2008; Peljto and Wichterle, 2011). Upper layer

neurons, those in layer II/III and IV are primarily callosal projection neurons (CPN) and granular neurons (GN). CPNs project their axons across the corpus callosum connecting the left and right hemispheres of the cerebral cortex. Deep layer neurons, in layers V and VI, are subcerebral projection neurons (SCPN) and corticothalamic projection neurons (CThPN) respectively. SCPNs extend axons towards the brainstem and spinal cord, while CThPNs target the thalamus, to relay sensory information to the cerebral cortex (Lodato et al., 2015). Although the subtypes of neurons in the cerebral cortex are well classified, when, and how neuron subtype is specified remains controversial and several models have been proposed.

The first model is that neuronal subtype is specified by temporal fate restriction of NPCs. Since neuronal subtype is highly correlated to neuronal birthdate, it is proposed that progressive restriction of NPCs dictates neuronal subtype. Indeed, previous transplantation experiments indicated that NPCs become temporally restricted (Desai and McConnell, 2000; McConnell and Kaznowski, 1991). That is, while early stage NPCs can form upper layer neurons when transplanted into later stage embryos, late-stage NPCs are unable to generate deep layer neurons when transplanted into earlier stage embryos (Desai and McConnell, 2000; McConnell and Kaznowski, 1991). This supports the model of neuronal subtype being specified pre-mitotically in NPCs. However, recent revalidation of these experiments shows that late-stage RGs, but not IPs, are competent to produce deep layer neurons when transplanted in the early embryonic cortices (Oberst et al., 2019). This disputes aspects of the temporal restriction model of subtype specification.

The second model is that NPC gene expression is temporally regulated, but not restricted, to assign neuronal fate. Fate mapping of individual progenitors indicates that a single progenitor can generate multiple neuronal subtypes, progressing from deep to upper layer neuron generation

(Shen et al., 2006). Further, the gene expression of NPCs has been shown to modify neuronal subtype. During pre-plate splitting, expression of Forkhead Box G1 (Foxg1) in NPCs suppresses generation of Cajal-Retzius cells, permitting layer VI neuron generation (Hanashima et al., 2004). β-catenin expression in NPCs also regulates laminar fate, with high levels of β-catenin promoting deep layer fate and restricting upper layer fate (Mutch et al., 2009). However, this model fails to fully explain how competing subtype specifiers can be co-expressed in NPCs, but give rise to a specific neuronal subtype (Hoye and Silver, 2021; Zahr et al., 2018).

The final model of subtype specification is that neurons are specified post-mitotically by environmental cues. Studies show that RGs express transcripts for both stem cell and neuronal fates prior to division, which may be subject to post-mitotic alterations (Telley et al., 2019; Yoon et al., 2017). One post-mitotic regulator is brain-derived neurotrophic factor (BDNF). It has been shown that increased levels of BDNF alter neuronal identity, promoting layer VI fate and restricting layer II/III generation (Fukumitsu et al., 2006). Neurotrophin 3 (Ntf3) expression from post-mitotic neurons influences progenitor fates, promoting upper layer neuron generation (Parthasarathy et al., 2014). Hyperpolarization of the cellular membrane of NPCs also changes neuronal progeny through inhibition of the Wnt/β-Catenin pathway (Vitali et al., 2018). Additionally, laminar positioning is thought to instruct and reinforce neuronal subtype through intercellular interactions (Castellani and Bolz, 1997; Chevée et al., 2018; Kwan et al., 2012). However, this model discounts the correlation between NPC gene expression and neuronal fate, and lacks evidence to account for the full spectrum of neuronal subtypes within the cortex.

Integration of these theories provides a more complete view of neuronal specification: that pre-mitotic regulation of NPCs cooperates with post-mitotic instruction of neurons (Albert and Huttner, 2018). For example, generation of upper layer neurons requires both transcriptional

repression of deep layer specifier T-box brain 1 (Tbr1), and feedback from post-mitotic deep layer neurons (Toma et al., 2014). This is consistent with the transcriptional priming of NPCs being modified post-mitotically by extrinsic signalling. Therefore, it is likely that neuronal subtype specification is regulated pre and post-mitotically, providing multiple layers of regulation to ensure proper development of the cerebral cortex.

Abnormal development of the cortex arising from disruption of neuronal subtype is associated with neurodevelopmental disorders (Arai and Taverna, 2017; Manuel et al., 2015; Martynoga et al., 2012). Indeed, many critical regulators of neuronal subtype are ASD associated genes (Ayhan and Konopka, 2019). It is unsurprising to find that in human patients as well as animal models of ASD, that cortical lamination and subtype identity is disrupted. For example, in animal models of ASD there is indistinct cortical lamination, and intermediary neuronal subtypes (Pucilowska et al., 2015). In studies of human ASD patients, patches of neuronal disorganization are found. These patches of disordered lamination show abnormal neuronal subtypes and may prevent functional integration of neuronal projections to adjacent columns and distant targets (Stoner et al., 2014). If the location and identity of these cells are altered, they cannot function in the required circuit (Martineau et al., 2018). As a result, understanding how neuronal subtype is specified is essential to understanding cortical development, and determining the effect of cortical malformations.

2.2 Transcriptional Regulation Networks

Transcriptional regulation is the first layer of control on gene expression. Transcription factors (TFs) use DNA binding sites to alter transcription of mRNA. TFs can act in a cross-repressive manner, where upregulation of one TF represses transcription of other competing TFs

(McKenna et al., 2011; Srinivasan et al., 2012). Cross-repression can occur directly, where a TF binds directly to the control region of another TF, or indirectly through secondary effectors (McKenna et al., 2011; Tang et al., 2011; Whitton et al., 2018). This competitive mechanism is used to control cell type in the developing cortex (Figure 3) (Leone et al., 2008; Srinivasan et al., 2012).

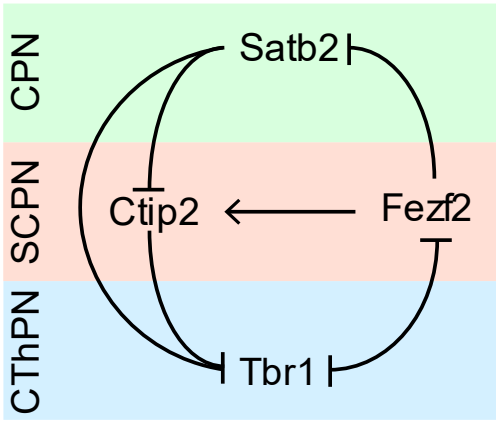

Figure 3. Simplified cross-repression network of transcription factors controlling neuronal subtype. Neuronal subtypes are established by cross-repressive molecular control. Undifferentiated neurons have overlapping subtype identity, and express multiple transcription factors. As neurons mature, subtypes become more distinct prevent expression of alternate transcription factors.

In neurons, transcription factor regulation is used to control neuronal subtype specification. Given the complexity of the cortex, the network of transcription factors involved in neuronal specification is large. Some of the major transcription factors known to regulate neuronal subtype specification in the cerebral cortex are Tbr1, B-cell lymphoma/leukemia 11B (Ctip2), FEZ Family Zinc Finger 2 (Fezf2) and Special AT-rich sequence-binding protein 2 (Satb2) (Figure 3) (Srinivasan et al., 2012). Loss of one or more of these transcription factors results in

complete loss of a neuronal subtype, altering the molecular and projection profile of the developing cortex (Arlotta et al., 2005; Bedogni et al., 2010; Dennis et al., 2017; Englund et al., 2005; Estivill-Torrus et al., 2002; Hevner et al., 2001; McKenna et al., 2011). These transcription factors are regulated spatially and temporally in the cerebral cortex (Alcamo et al., 2008; Bedogni et al., 2010; Dennis et al., 2017; Englund et al., 2005; Estivill-Torrus et al., 2002; Greig et al., 2013; Hanashima et al., 2004; Hevner et al., 2001; Oishi et al., 2016; Srinivasan et al., 2012; Wiegreffe et al., 2015).

Spatially, Tbr1 expression levels are high in the frontal cortex, and decrease caudally. Tbr1 knockout results in both caudalization of the developing cortex, and conversion of presumptive layer VI corticothalamic projection neurons (CThPN) neurons to a layer V sub cerebral projection neuron (SCPN) fate (Bedogni et al., 2010). Tbr1 does this through direct repression of SCPN specifier Fezf2, as shown by chromatin immunoprecipitation (CHIP). While Tbr1 is known to bind the Fezf2 gene directly, Fezf2 also restricts Tbr1 expression (McKenna et al., 2011). This restriction of Tbr1 is essential to allow the transition from layer VI to layer V generation at E13.5 (Dennis et al., 2017; McKenna et al., 2011). Loss of Fezf2 results in overabundance of Tbr1+ CThPNs, and loss of Ctip2+ SCPNs.

Loss of SCPNs in Fezf2 knockouts is due to the loss of Ctip2 expression. Ctip2 is downstream of Fezf2 and specifies layer V SCPNs. Reintroduction of Ctip2 restores SCPN fate and rescues subcerebral projections within the cortex (Chen et al., 2008). Fezf2 knockout mice also show an increase in upper layer callosal projection neurons (CPNs) as a result of increased Satb2 expression.

Satb2 expression is repressed by TFs Fezf2, Neurog2 and Ascl1 in early development, preventing premature generation of CPNs in the cortex (Dennis et al., 2017). Satb2 represses

Ctip2 expression directly, driving upper layer neuron generation (Alcamo et al., 2008; Dennis et al., 2017; Whitton et al., 2018). Precocious expression of Satb2, by Neurog2/Ascl1 double knockout, results in ectopic expression of Satb2+ neurons through the cortex, reducing deep layer neuron generation. Loss of Satb2 does not impact Tbr1+ deep layer CThPNs, but shows expansion of Ctip2+ cells into the upper compartments of the cortical plate (Alcamo et al., 2008).

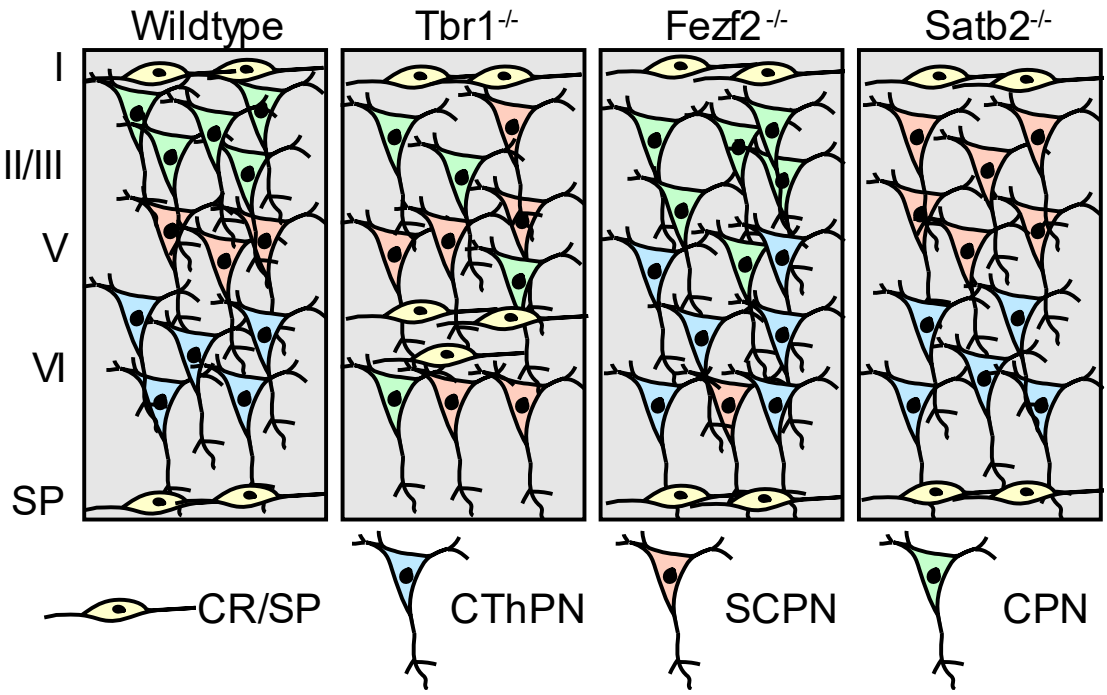

Figure 4. Loss of neuronal transcriptional factors alters cortical structure. Schematic representation of transcription factor knockout phenotypes in the cortex. Cajal-retzius cells (CR) and subplate neurons (SP) (yellow), CThPN (blue), SCPN (pink), CPN (green). Loss of Tbr1 disrupts subplate formation and CThPN generation (Bedogni et al., 2010). Fezf2 knockout alters SCPN formation and localization, increasing CThPN and CPN generation (Chen et al., 2008). Satb2 knockout prevents CPN formation, resulting in abnormal SCPN formation (Srinivasan et al., 2012).

Together, this web of transcription factors is essential for mediating the timing and identity of developing cortical neurons (Alcamo et al., 2008; Bedogni et al., 2010; Dennis et al., 2017; Greig et al., 2013; Oishi et al., 2016; Woodworth et al., 2016). However, these represent only a small subset of all transcription factors in the developing cortex. The interconnected nature of TFs makes their regulation complex, and highly susceptible to secondary regulation. This regulation can be triggered extrinsically by signalling molecules, or intrinsically through post-transcriptional regulation.

2.3 Post-transcriptional control of RNA

Post-transcriptional control can alter the translational profile of the cell. This is done by altering the bio-availability of mRNAs, which tailors protein expression. Post-transcriptional control can occur at any point between transcription and translation. For example, mRNA transcripts undergo extensive modification and transport following transcription from DNA (Moore and Proudfoot, 2009). Newly transcribed pre-RNA requires addition of a 5' cap, splicing, 3' poly-adenylation to be converted to mature mRNA. Processed mRNA must then be shuttled from the nucleus to the cytoplasm for translation. Once mRNA is in the cytoplasm it is subject to further modification, such as extension of the poly-A tail (Mata et al., 2005). This extensive processing and transport provide multiple points for regulation prior to mRNA being available for translation.

Following the maturation of mRNA, cytoplasmic mRNAs are subject to translational regulation. Translational regulation can either enhance, or repress RNA translation. The primary executor of mRNA regulation are RNA binding proteins (RBPs). RBPs bind RNA directly using either sequence specific target sites or secondary RNA structure, such as hairpin folds (Hentze et

al., 2018; Mata et al., 2005). RBPs can act either directly, through catalytic domains, or as adaptors. As adaptors RBPs allow mRNA interaction with additional proteins either directly, or indirectly (Figure 5). This allows RBPs to bind a multitude of mRNA targets and be recruited to a number of different protein complexes, such as processing bodies (PBs) and stress granules (SGs) to form ribonucleoprotein (RNP) complexes. As a result, binding of RBPs does not act on mRNA targets uniformly – some targets may be translationally enhanced by increasing stability and ribosome recruitment, while others may be targeted for degradation or repressed through sequestration (Hentze et al., 2018). This is variable nature of RNA binding is invaluable for post-transcriptional control within the cell, however the complexity of RBP-RNA obscures the effects of single RBPs: the same RBP may affect two different RNA targets in opposite ways.

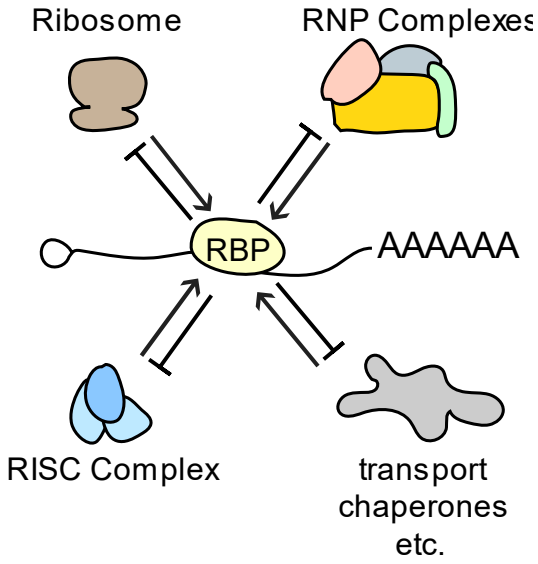

Figure 5. RNA binding proteins act to modify RNA bioavailability. RBPs can reversible bind and release RNA targets in the nucleus or cytoplasm. RBPs may act directly to modify target availability or interact with additional complexes. This includes preventing or enhancing binding of ribosomes, RNP complexes, the RISC complex, and other proteins.

Despite its importance, our understanding of translational control in the developing brain is limited. Research has primarily focused on translational control in stem cells. For example, during corticogenesis, self-renewal and differentiation of stem cells is altered post-transcriptionally (Ju Lee et al., 2017; Rodrigues et al., 2016, 2020; Yang et al., 2014; Zahr et al., 2018). In human embryonic stem cells (hESCs), differentiation is suppressed by translational repression of Methyl CpG-binding protein 2 (MECP2), a transcription factor crucial for normal brain development. *MECP2* is repressed by binding of stem cell RBPs, resulting in mRNA degradation. This persists until neuronal RBPs are expressed, replacing stem cell related RBPs to promote *MECP2* translation and drive hESC differentiation (Rodrigues et al., 2016).

In NPCs, Notch signalling maintains NPC fate, supressing differentiation. *Notch1* mRNA is subject to translational repression by RNA binding protein GAPDH. This repression of *Notch1* occurs when high levels glycolytic metabolites accumulate in the cell. This drives *Notch1* inactivation, increasing pro-neural gene expression (Rodrigues et al., 2020). Additionally, NPC fate is maintained by RNA processing bodies. These P-bodies show enrichment for neural transcription factors and pro-differentiation mRNAs, sequestering them from translation machinery. This prevents premature neurogenesis and NPC depletion in the cortex (Yang et al., 2014).

During neurogenesis, RBP Pumilio2 (Pum2) acts in RGs to repress translation of neuronal identity mRNAs through interaction with PB protein Eif4enif1 (4-ET). This repression of subtype specific identity genes ensuring appropriate subtype specification of cortical neurons (Zahr et al., 2018). In addition to the defined role of translational control during self-renewal and neurogenesis, disruption of key RNAs and RNA processing centres is sufficient to cause neurodevelopmental defects (Ju Lee et al., 2017; Rodrigues et al., 2016, 2020; Yang et al., 2014;

Zahr et al., 2018). Despite the evidence that RNA metabolism is crucial to NPC maintenance and neurogenesis, our understanding of translational regulation in the developing cortex is incomplete. Particularly, our understanding of RBPs and translational regulation as it relates to neuronal subtype specification is sparse.

2.4 RNA-binding protein Csde1

To investigate the role of RBPs in corticogenesis, I used a single RBP approach. By this approach, a single candidate gene is used as a tool to dissect a larger process. In this case a successful candidate would cause neurodevelopmental disorders when disrupted, allowing us to examine the underlying pathology of the disorder. To select a candidate, I examined known RBPs in the developing cortex and focused on genes in which mutations cause neurodevelopmental disorders. From these candidates I chose RBPs primarily located in the cytoplasm, where translational control is most active. Using these parameters, I isolated Cold shock domain containing E1 (Csde1).

Csde1 is an 89kDa cytoplasmic protein with five canonical and four non-canonical cold shock domains (Hollmann et al., 2020; Jacquemin-Sablon et al., 1994). Csde1 is expressed in all tissue types, including the cerebral cortex (Uhlén et al., 2015).Csde1 has defined RNA binding capability, and is known to target purine-rich single stranded nucleic acids, stem-loop structures and internal ribosome entry sites (IRES) through its five canonical cold-shock domains (Anderson and Catnaigh, 2015; Anderson et al., 2007; Hollmann et al., 2020; Militti et al., 2014; Moore et al., 2018a; Saltel et al., 2017). Csde1 target specificity is mediated by its 4 non-canonical cold-shock domains, in addition to secondary protein interactions. For example, Csde1 binding to scaffolding protein STRAP alters Csde1 RBP activity (Moore et al., 2018b).

Csde1 can act independently to alter RNA translation, or as an RNA adaptor, bringing target mRNAs in contact with RNP complexes (Anderson and Catnaigh, 2015; Guo et al., 2020; Mitchell et al., 2003; Youn et al., 2018). The variable nature of Csde1 is best exemplified in *Drosophila melanogaster*, where Csde1 acts as critical component of the X-chromosome dosage compensation mechanism (Abaza et al., 2006; Duncan et al., 2006, 2009; Hennig et al., 2014; Militti et al., 2014). In females, interaction of sex specific protein, sex-lethal (SXL), targets Csde1 to the 3' UTR of male sex-lethal 2 (msl-2). Csde1 3' UTR binding represses translation of msl-2, an essential component of the dosage compensation complex (DCC), thereby preventing DCC formation in females (Duncan et al., 2006). Interestingly, Csde1 is also essential for the function of the DCC in males. Loss of Csde1 reduces DCC interaction with the X-chromosome (Patalano et al., 2009). The DCC complex is an RNP complex, which uses long non-coding RNAs (lncRNAs), *RNA on X (roX) 1* and *2*, to target the X-chromosome. Csde1 binds stem-loop structures of *roX2*, mediating the assembly the DCC on the male X-chromosome (Militti et al., 2014). Although mammalian dosage compensation occurs through different mechanisms, the role of Csde1 in *D. melanogaster* dosage compensation is an example of the variable nature of Csde1 RNA binding.

Csde1 is known to interact several RNA processing complex proteins (Hollmann et al., 2020; Kakumani et al., 2021; Youn et al., 2018). Interactors of Csde1 include splicing proteins Strap, Nito and Rump (King et al., 2014; Lence et al., 2016; Moore et al., 2018b), RNA-induced silencing complex (RISC) protein Ago1 (Okamura et al., 2004), mRNA stability mediator AUF1 (Dinur et al., 2006), major processing body (P-body) and stress granule (SG) component DDX6 (Kamenska et al., 2016), as well as RNA binding proteins PTB and PABP (Anderson and Catnaigh, 2015; Chang et al., 2004). As seen in with SXL, interaction of secondary proteins can

modify Csde1 activity. This makes determination of Csde1 mRNA targets difficult, and masks potential mechanisms of Csde1 control.

Despite the elusive nature of Csde1 mRNA targets, it is apparent that Csde1 is an important regulatory factor across cell types. To this end, Csde1 itself is auto regulatory: Csde1 can repress or enhance translation of its own mRNA, and may monitor its own expression level (Cornelis et al., 2005; Dormoy-Raclet et al., 2005; Schepens et al., 2007). This translational regulation allows quick modification of Csde1 protein level in the cell. For example, in human embryonic kidney (HEK293) cells during mitosis, Csde1 is upregulated. This upregulation is specific to the G2/M transition, and allows Csde1 to interact with secondary RNA binding protein hnRNP C1/C2, to increase translation of CDK11A-p58. This increase of CDK11A-p58 helps drive mitosis (Schepens et al., 2007).

In erythroblasts, Csde1 is highly expressed and interacts with mRNAs associated with RNA processing and translation (Moore et al., 2018a). Independently, the effect of Csde1 modification in these cells is modest. Within these cells however, Csde1 interacts with STRAP, a scaffolding protein that has no inherent RNA binding capability. Through its interaction with STRAP, a subset of Csde1 bound transcripts are differentially regulated, namely Vimentin, Gata-1 and Elavl1 (Moore et al., 2018b). These transcripts are associated with erythrocyte maturation, and hematopoietic differentiation.

The diverse regulation of mRNAs associated with Csde1 mean that disruption of Csde1 can have wide reaching effects within a cell. Indeed, mutations in Csde1 are associated with disease including diamond-blackfan anemia (DBA) and cancer, as well as neurodevelopmental disorders such as ASD (Guo et al., 2020, 2019; El Khouri et al., 2021; Moore et al., 2018a).

Diamond-blackfan anemia is a genetic blood disorder, caused by disruption to red blood cell production from the bone marrow (Ball, 2011). There are multiple genetic mutations responsible for DBA, including mutations in Gata-1, which affect hematopoietic differentiation. Csde1 is known to affects Gata-1 translation in erythroid cell types (Moore et al., 2018b). Additionally, Csde1 expression is reduced in DBA, and is associated with impaired proliferation and differentiation of hematopoietic cell types (Horos et al., 2012).

Studies show Csde1 as a prognostic marker in multiple types of cancer. In melanoma, glioma and colorectal cancer, overexpression of Csde1 promotes migration and metastasis (Kakumani et al., 2021; Martinez-Useros et al., 2019; Tian et al., 2018; Wurth et al., 2016). It does this through upregulation of pro-oncogenic targets such as c-Myc, vimentin and RAC1 (Martinez-Useros et al., 2019; Wurth et al., 2016). In pheochromocytoma and paraganglioma, rare types of adrenal cancer, Csde1 mutations are associated increased metastatic potential as a result of altered Wnt signalling (Fishbein et al., 2016, 2017).

Wnt signalling is also known to be disrupted by an ASD associated mutation of Csde1. In human patient derived fibroblasts with a nonsense mutation in Csde1, shows inhibition of the Wnt signalling pathway by increased translation of APCDD1. Additionally, these cells show enhanced cellular adhesion through increased expression of Cadherin2 (El Khouri et al., 2021). Supporting the association of Csde1 with ASD, other variants of Csde1 are also associated with ASD (Guo et al., 2019). Previous work shows that Csde1 is expressed in hESCs, neuroepithelial cells and pre-cerebellar neurons, indicating its expression in the developing brain (Ju Lee et al., 2017; Kobayashi et al., 2013). In stem cells, Csde1 restricts the transition of pluripotent hESCs to committed neuroepithelial lineages (Ju Lee et al., 2017). In precerebellar neurons, Csde1 is required for both tangential and radial migration and Csde1 deficient neurons fail to migrate to

their final position (Kobayashi et al., 2013). Additionally, Csde1 deficient neurons show decreased axon growth, decreased arborisation and reduced synaptic function (Guo et al., 2019). Analysis of the mRNA targets of Csde1 show binding affinity for other ASD associated genes (Guo et al., 2019; Xia et al., 2014). Together, this indicates that Csde1 may be crucial to development of neural cell types.

Given the dynamic nature of Csde1 and its influence in key developmental processes from mitosis to cell maturation, determining how Csde1 influences the cortical environment provides an inroad for understanding translational regulation in cortical development. Translational programs coordinated by Csde1 influence cell cycle dynamics, cell differentiation and cell migration. These are all essential processes during corticogenesis, and Csde1 therefore provides a candidate for understanding translational regulation in corticogenesis.

Chapter 3: Materials and Methods

3.1 Mice

All animal use was approved by the Animal Care Committee at the University of Calgary. Animal care and use was completed in accordance with animal use protocols AC17-0099 and AC21-0026. CD-1 and C57Bl/6 mice were purchased from Charles River Laboratory. Transgenic $Csde1^{tm1a(EUCOMM)Wtsi}$ mice in the C57Bl/6 background were purchased from European Mutant Mouse Archive (EMMA) and crossed to obtain $Csde1^{tm1e/+}$ (Figure 14A). $Csde1^{tm1e/+}$ mice contain a tm1e allele which uses a LacZ gene trap to prevent Csde1 expression. $Csde1^{tm1e/+}$ will be referred to as $Csde1^{KO/+}$ for the remainder of this document. Presence of tm1e cassette was assessed using PCR amplification genotyping.

3.2 Timed Mating

To determine embryonic age for experiments female mice were placed with a single male overnight and checked for a copulatory plug the following morning. Presence of a copulatory plug was designated as embryonic day (E) 0.5.

3.3 Neural Progenitor Cell Culture

To culture primary neural progenitor cells, pregnant CD-1 mice were euthanized by cervical dislocation at E12.5. Embryos were harvested and placed in ice cold Hank's Balanced Salt Solution (HBSS) to ensure cell viability. The cortex was then dissected from each embryo and placed in ice cold supplemented Neurobasal media. Neurobasal Media (Gibco #21103-49) was supplemented with 40 ng/mL Fibroblast Growth Factor 2 (FGF2) (Thermo Fisher Scientific #CB-40060A), 0.5 mM L-Glutamine (Sigma #56-85-9), 2% B-27 (Thermo Fisher Scientific #17504044), and 1% Penicillin-streptomycin solution (Wisent #450-201-EL).

Cells were titurated using a plastic Pasteur pipette, then passed through a 40µm strainer to obtain a single cell suspension. Cells were counted using a hemocytometer using trypan blue staining. Cells were plated at a density of 125,000 cell/mL onto rinsed pre-coated coverslips. Coverslips were pre-coated with 5µg/mL laminin (VWR #CACB354232) and 1mg/mL poly-D-lysine (VWR #P6407) and incubated overnight at room temperature.

3.4 Transfection of NPC cultures

Neural stem cells (NSCs) were transfected 4hours following plating. 1µg DNA total was transfected using 2µL lipofectamine stem reagent (Fisher #STEM00008) and topped up to 50µL using opti-MEM (Thermofisher #31985070). Cells co-transfected with GFP vector for tracking were transfected at a ratio of 3:1 (Construct:GFP). This mixture was incubated at room temperature for 30 minutes, then added to the cells. These cells were incubated at 37°C for three days following transfection.

3.5 Preparation of cortical sections

To analyze neurons *in vivo,* whole mount brains were prepared for immunohistochemistry (IHC). Pregnant mice (E15.5 or E18.5) were euthanized by cervical dislocation and embryos were harvested into ice cold HBSS. Postnatal mice were euthanized by decapitation seven days after birth (P7). Both embryonic and postnatal mice were dissected and the whole brain was placed in 4% paraformaldehyde (PFA) overnight at 4°C. Following overnight incubation, brains were removed from PFA and placed in a solution of 30% sucrose dissolved in phosphate buffered saline (PBS) for cryopreservation overnight at 4°C. Cryopreserved brains were placed in disposable base molds and covered with Frozen Section Compound (VWR 95057-838) and stored at -80°C prior to sectioning. Frozen mounted brains were sectioned at 16µm using a cryostat (Leica CM1950) to collect samples of the somatosensory cortex.

3.6 Immunostaining

Cortical sections and NPC cultures were processed using immunofluorescent (IF) staining. Primary and secondary antibodies used varied by application. All antibodies used are summarized below.

Target	**Species**	**Company**	**Catalogue number**	**IHC Concentration**
Pou3f3 (Brn1)	Goat	Novus	NBP1-49872	1:500
Satb2	Mouse	Abcam	ab51502	1:1000
Ctip2	Rat	Abcam	ab18465	1:1000
Tbr1	Rabbit	Abcam	ab31940	1:1000
Csde1	Rabbit	Abcam	ab201688	1:1000
TuJ1 (B-III)	Mouse	Biolegend	801201	1:1000
Sox2	Goat	R & D	AF2018	1:1000
GFP	Chicken	millipore	AB16901	1:1000
4-ET	Mouse	Abnova	H00056478	1:100
Rabbit 488	Donkey	Thermo	A32790	1:1000
Mouse 488	Donkey	Thermo	A32766	1:1000
Rat 555	Donkey	Thermo	A32794	1:1000
Mouse 555	Donkey	Thermo	A32773	1:1000
Rat 555	Donkey	Thermo	A48270	1:1000
Goat 555	Donkey	Thermo	A32816	1:1000
Rat 647	Goat	Thermo	A-21247	1:1000
Mouse 647	Donkey	Thermo	A32787	1:1000
Goat 647	Donkey	Thermo	A32849	1:1000

Table 2. Antibodies used in Immunohistochemistry. Antibodies and working concentrations for primary and secondary antibodies. Primary antibodies of differing species were used in combination to co-stain multiple proteins.

3.6.1 Cortical Sections

Sectioned cortical samples were stored at -80°C until staining. Slides were washed three times with PBS to remove frozen section compound. Sections were blocked and cells permeabilized using IHC blocking buffer (5% BSA, 0.2% triton-X in PBS) for 1 hour at room temperature. Primary antibodies were diluted in a 1:1 IHC blocking buffer:PBS solution, and incubated at 4°C

overnight. Following primary antibody incubation slides were washed three times with PBS. Secondary antibody diluted in PBS was incubated at room temperature for 1 hour. Slides were washed three times with PBS, and counterstained with Hoechst 33258 (Thermofisher #H3569) diluted 1:5000 in PBS. Slides were washed three times with PBS following staining and mounted using Aqua-Poly/Mount (Polysciences #18606-20) and a coverslip sealed with clear nail varnish.

3.6.2 NPC Cultures

Coverslips were washed three times with PBS to remove remaining media. Cells were fixed using 4% PFA at room temperature for 20 minutes. Following fixation, cells were permeabilized using 0.2% Triton-X in PBS for 3 minutes. Coverslips were washed three times with PBS to remove any remaining Triton-X and blocked with 5% Normal Donkey Serum (NDS) in PBS for 30 minutes. Blocking buffer is removed and primary antibody diluted in 5% NDS is incubated 1 hour at room temperature. Coverslips are washed three times in PBS and incubated 1 hour with secondary antibody in 5% NDS. Cells are counterstained with Hoechst and washed 3 times with PBS prior to mounting.

3.7 *In situ* Hybridization

RNAScope Multiplex FiSH system (ACD Bio #320850) was used for Fluorescence *in situ* hybridization (FISH). Frozen section compound was dissolved using PBS and slides post-fixed in 4%PFA for 10mins at RT. Sections were desiccated using progressive ethanol washes: 50% EtOH 1min, 70% for 1 minutes, 100% for 3 minutes, 100% for 3 minutes. Slides were air dried for 15 minutes following ethanol washes. Cells were permeabilized using PBS and PBS-T (PBS with 0.1% Triton-X) for 10 minutes and rinsed with PBS. Prepared probe was added for 2 hours at 40°C in a humid chamber then washed 4x with RNA Scope wash buffer. Probes were amplified using Amp1 for 30 minutes, Amp2 15 minutes and Amp 3 for 30 minutes at 40°C.

Amplified probes were incubated with fluorescent probe Amp4-FL in appropriate channel 15 minutes 40°C. Slides were washed wish wash buffer between each amplification step. Sections counterstained with Hoescht 1 minute RT, washed three times with PBS and mounted using Aqua-Poly/Mount (Polysciences #18606-20) and a coverslip sealed with clear nail varnish.

3.8 PCR

3.9 Primer	**Purpose**	**Sequence**
Csde1_5arm_neu	Genotyping	GTAGGAGGTAATCTGTCAGCT
Csde1_3arm_neu	Genotyping	ATCTTGCATCTGACCTCTAAG
CAS_R1_term	Genotyping	TCGTGGTATCGTTATGCGCC
GAPDH mouse – F	qRT-PCR	ACCACAGTCCATGCCATCAC
GAPDH mouse – R	qRT-PCR	CACCACCCTGTTGCTGTAGCC
Csde1-qRT-ex-2F	qRT-PCR	CTTGGTAGATCATTTCCTCTCGC
Csde1-qRT-ex-2R	qRT-PCR	GTCCATTGTTGTGGAGAAGGT

Table 3. **Primer Sequences for genotyping and qRT-PCR.** All sequences shown 5'- 3'.

3.8.1 Genotyping

Mice carrying the Csde1^{tm1e} allele were identified using PCR genotyping. Using forward and reverse primers specific to the mutant cassette, amplification of PCR product can only occur when the Csde1^{tm1e} allele is present. Adult mice were genotyped using ear tissue from the ear identification marking (see Animal protocol). Embryonic mice were genotyped using skin tissue removed during cortical dissection.

DNA extraction of tissue samples was done using KAPA express extract kit (KK7100). PCR reaction done using DreamTaq Green PCR Master mix (K1081). PCR products analyzed by agarose gel electrophoresis on 0.8% agarose gel to detect presence of amplicon.

3.8.2 Quantitative Real Time PCR (qRT-PCR)

Embryonic cortices were obtained by dissection and RNA extracted using E.Z.N.A. total RNA kit (Omega #R6834). cDNA was synthesized from total RNA using Maxima cDNA synthesis kit (Fisher #m1681). PerfeCTa SYBR Green FastMix (Quanta #95072-012) was used for the qPCR reaction. qRT-PCR analysis was done using CFX Connect Real-Time PCR System (Bio-Rad 1855200) and ΔCT method compared to GAPDH housekeeping gene.

3.10 Western Blot

Embryonic cortices were obtained by dissection and lysed using tissue lysis buffer (150mM NaCl, 20mM Tris, 1mM EDTA, 1mM EGTA, 1% Triton-X). Lysates were incubated on ice for 10 minutes then centrifuged at 10,000g for 10 minutes at 4°C to remove cellular debris. Cleared supernatant was removed and mixed with 4x Laemmli buffer and heated at 95°C for 10 minutes.

Transfected cells were washed 1 time with PBS, lysed using 4x Laemmli buffer and heated at 95°C for 10 minutes.

All lysates were run using polyacrylamide gel electrophoresis (PAGE) at 100V for 55 minutes. Proteins were transferred to nitrocellulose membranes for 2 hours at 100V. Membranes were blocked for 1 hour at room temperature using 5% BSA in TBS-T and incubated with primary antibody diluted in 5% BSA in TBS-T overnight at 4°C. Following primary antibody incubation, the membrane was washed three times for 10 minutes with TBS-T. Membrane was then incubated with HRP-conjugated secondary antibody (Invitrogen #a16023) in PBS for 1 hour at room temperature. Membrane was washed three times for 5 minutes with TBS-T, analysed using ECL substrate (Sigma #RPN2109) and imaged for chemiluminescence (Amersham Imager 600). Images quantified using ImageJ.

3.11 Microscopy and Quantification

Imaging was done using an Olympus FV3000 confocal microscope. Processing and quantification were done using ImageJ. Cortical sections were stitched together from multiple images using a plugin developed by Preibisch *et al.* (Preibisch et al., 2009)

For neurogenesis and subtype distribution, cells were counted using ImageJ cell counter. 3 columns were taken from each image and counted independently, then averaged prior to analysis. Cell counts were exported as X,Y coordinates. Using these coordinates, the total size of the cortical plate was determined by Hoechst staining, and divided into 15 equal divisions, or bins. These bins were used as a reference for marker positive cells. The total number of marker positive cells per bin was summed, and the relative distribution was determined by dividing the sum of each bin by the total number of marker positive cells in the overall cortical plate.

3.12 Statistical analysis

In NPC cultures, two-tailed unequal variance t-tests were performed comparing the mean proportion of marker positive cells across 4 trials. To compare between wildtype and $Csde1^{KO/+}$ groups a two-tailed, equal variance, students t-test was used. For overall cortical size and cell number, the counted values were compared directly. For cortical distribution, a t-test was performed for each bin, to compare the mean distribution of each bin. Error bars represent standard error.

Chapter 4: Expression of Csde1 in the developing cortex

To determine if Csde1 is expressed during corticogenesis, I first examined Csde1 expression *in vitro* using primary NPC culture. I determined that Csde1 is expressed in both NPCs and immature neurons. Additionally, I show that Csde1 co-localizes with P-bodies, which regulate RNA metabolism (Luo et al., 2018; Youn et al., 2018). I also examined Csde1 expression *in vivo* using cortical sections and found that Csde1 is spatially regulated within the cortex.

4.1 Csde1 is expressed in neural progenitor cells and neurons

To determine the expression of Csde1 in cultured NPCs, I dissected cortices from the mouse embryos at the embryonic day (E) 12.5, when neurogenesis begins in the cortex. Cells were cultured for three days. Immunostaining for the NPC marker Sox2 (SRY-box 2) and the neuronal marker β-III tubulin confirmed the successful isolation and culture of cortical cells (Figure 6). I then examined the expression of Csde1 using a commercially available antibody. The specificity of this antibody was validated by knocking down Csde1 in cortical cultures (see Chapter 5). Co-immunostaining showed that Csde1 was robustly detected in both Sox2+ NPCs and β-III tubulin+ neurons (Figure 6). Importantly, Csde1 was predominantly detected in the cytoplasm of both NPCs and neurons, consistent with its biological role as a cytoplasmic mRNA regulator. Further examination of Csde1 expression revealed distinctive granular structures in the cytoplasm, raising the possibility that Csde1 is present in processing bodies (P-bodies), cytoplasmic organelles critical for mRNA metabolism.

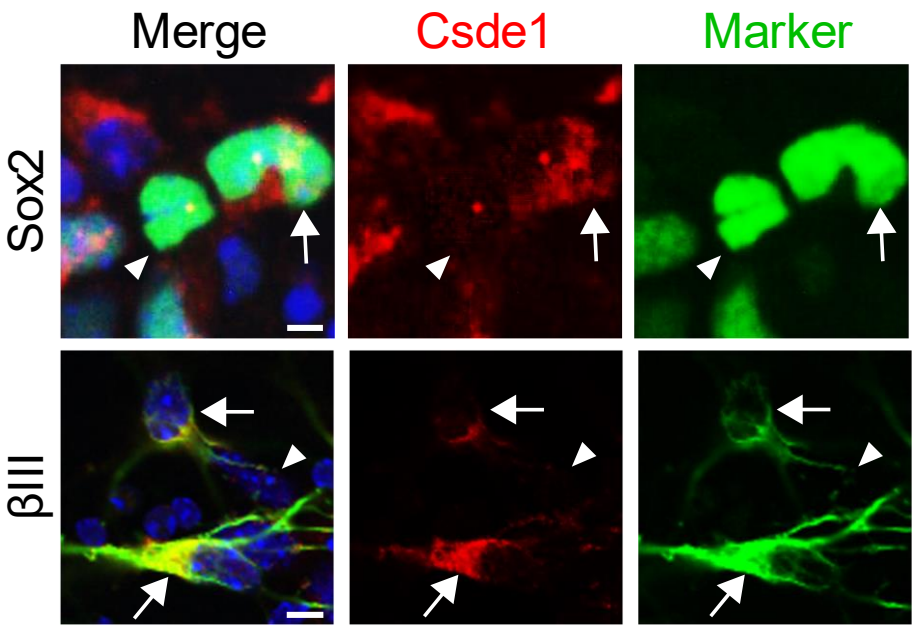

Figure 6. Csde1 is expressed in NPCs and Neurons. Expression of Csde1 (red) in NPC cultures. High (arrows) and low (arrowheads) expression of Csde1. Stem cells marked by Sox2 (top) and immature neurons marked by β-III tubulin (bottom). Counterstained with Hoechst (blue). Scale bar = 10μm.

To test this hypothesis, I performed immunostaining for a P-body marker, eIF4Enif1 (4E-T) and found that Csde1 was colocalized with 4E-T, confirming its presence in P-bodies (Figure 7). However, I also detected abundant Csde1 present outside of P-bodies, suggesting that Csde1 may function in manners both related and unrelated to P-bodies (Figure 7).

Given that Csde1 is expressed in cultured NPCs and neurons and can be recruited to RNA P-bodies, it indicates that Csde1 is active in the RNA metabolism *in vitro*. I next wanted to determine if Csde1 expression was observed *in vivo*.

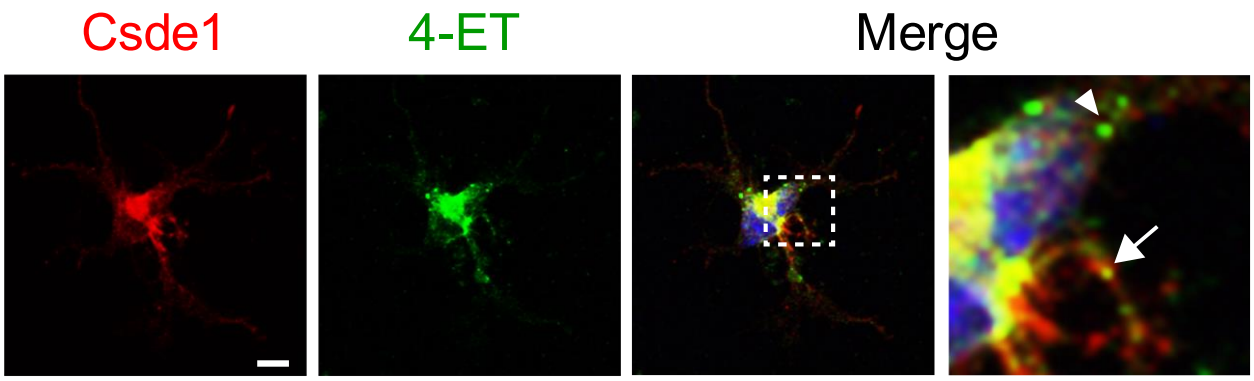

Figure 7. Csde1 co-localizes with RNA complex proteins. Primary NPC culture. Csde1 co-localizes to P-Body component 4-ET (arrow). Csde1 is expressed outside of 4-ET puncta (arrowheads). Enlarged image (right). Counterstained with Hoechst (blue) Scale bar = 10µm.

4.2 Csde1 is expressed in the developing Mouse Cortex

I next assessed the expression of *Csde1 in vivo* in the developing cortex. The embryonic cortex was micro-dissected from mice at different gestational stages. These embryonic cortices were used to assess RNA and protein expression. Csde1 mRNA levels were determined by quantitative PCR (qPCR). I found that levels of Csde1 increased through embryonic development, peaking during late development. Expression of Csde1 mRNA increased by approximately 4-fold by E18.5, compared to the early E12.5 cortex ($p<0.001$). Post-natal levels (postnatal day (P) 5) of mRNA were consistent with late embryonic levels (E18.5) (Figure 8A**).** Protein level of Csde1 showed a similar increase in expression during development. Although protein levels increased in late embryonic and post-natal cortices, the magnitude of change was reduced compared to mRNA levels (Figure 8C). Csde1 protein expression increased approximately 1.5-fold between E12.5 and E18.5 ($p=0.03$). This difference in magnitude

between mRNA and protein expression, indicates that Csde1 protein expression may be altered at the post-transcriptional level.

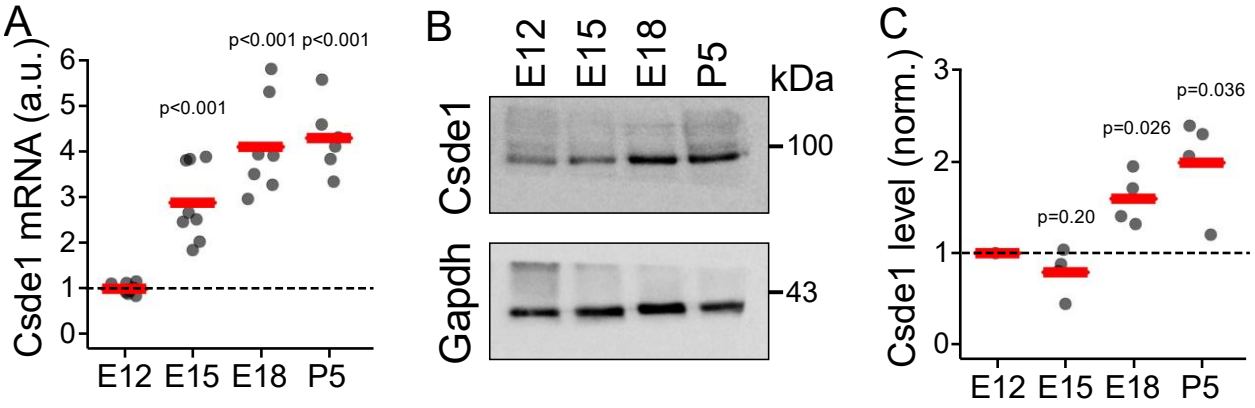

Figure 8. Csde1 expression increases during development. Expression of Csde1 mRNA by qRT-PCR analysis from E12.5 to P5. Expression was normalized to average expression at E12. n=6-8, represented by dots. (B) Protein expression of Csde1 by western blot. (C) Quantification of Csde1 protein expression by western blot. Relative expression compared to Gapdh loading control was normalized to relative expression at E12.5. n=4, represented by dots. Two-tailed students t-test.

Given the discordance of mRNA and protein levels, I assessed the spatial expression pattern of Csde1 during development. To this end, I used molecular markers of neuronal subtype to determine the lamination of the cortex in embryonic coronal sections. Using upper layer marker Brn1 (POU Class 3 Homeobox 3, Pou3f3) and layer V marker Ctip2 (B-cell lymphoma/leukemia 11B, Bcl11b) I can approximate the boundaries of cortical layers. These

markers provide a reference for more accurate determination of Csde1 expression patterns when co-stained within the cortex.

Immunofluorescent staining (IF) shows that Csde1 expression was low throughout the E15.5 cortex (Figure 9). There were some Csde1 positive cells in the VZ and the cortical plate, although Csde1 was only high in individual cells within these compartments. Csde1 expression was high in upper layer Brn1+ cells within the CP. Csde1 was also expressed in Ctip2+ migrating cells within the IZ, but showed lower expression in Ctip2+ cells in the CP.

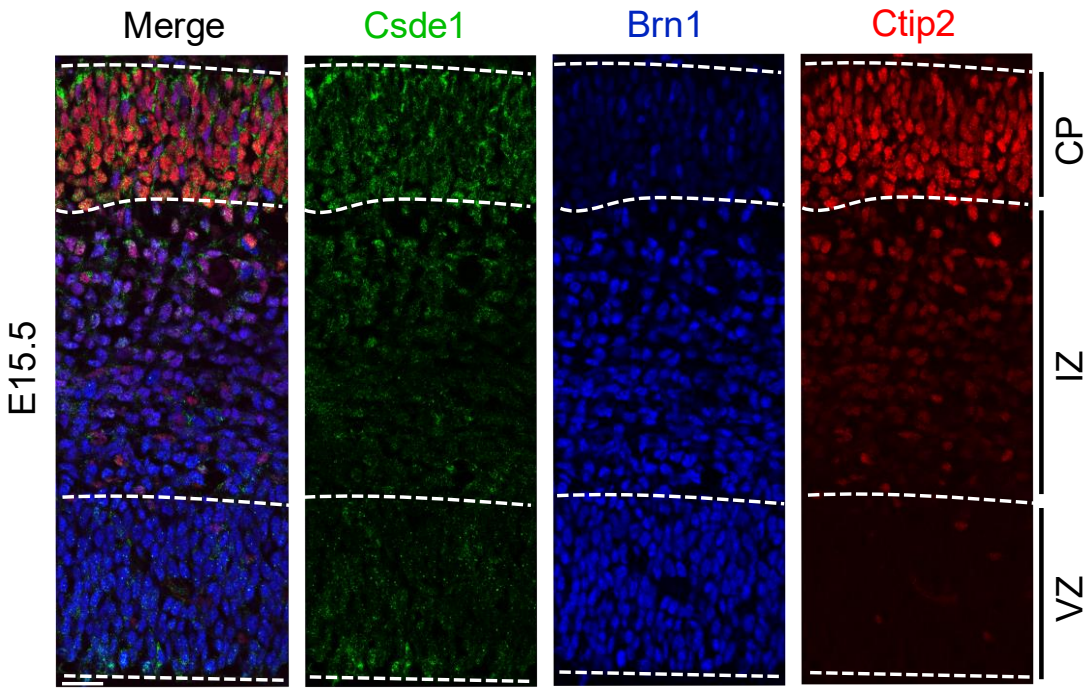

Figure 9. Csde1 Protein expression in the cortex at E15.5. Coronal section in CD-1 mouse line. Cortical region denoted by dotted line. Scale bar = 50µM

During the late embryonic stage (E18.5) as observed in protein and mRNA analysis, IHC also shows high Csde1 expression in the cortex (Figure 10). Csde1 expression was restricted to the upper layers of the cortex, with expression dropping off in mid layer V, marked by Ctip2 expression. This Csde1 expression was seen in both Brn1+ and Ctip2+ cells within these areas. Expression of Csde1 was low in layer VI and appeared as punctate points. This punctate expression was variable in density in layer VI, and was also visible in the subplate. Csde1 immunosignal was not seen in the IZ or in the VZ, which showed very low expression (Figure 10).

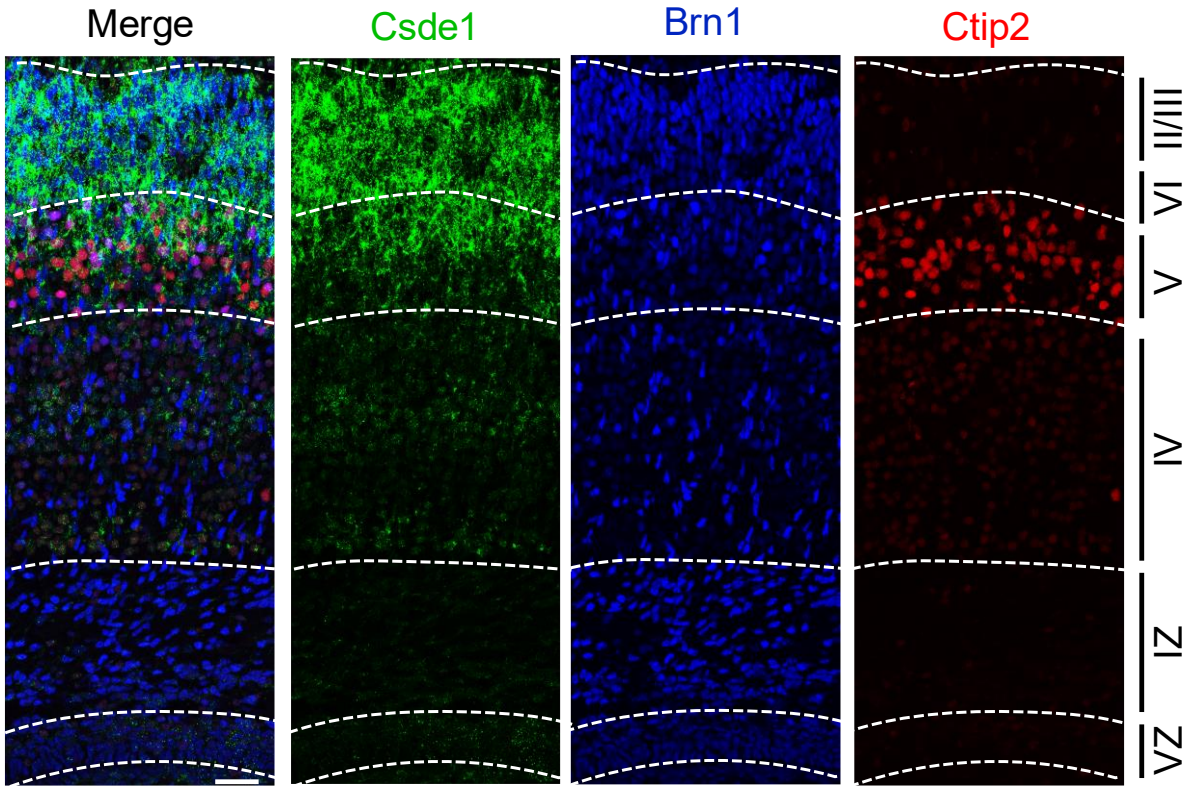

Figure 10. Csde1 Protein expression in the cortex at E18.5. Coronal section E18.5. Cortical region denoted by dotted line. Scale bar = 50µM

To determine if the restricted pattern of Csde1 protein expression was regulated at the transcriptional or post-transcriptional level, I next wanted to compare the localization of *Csde1* mRNA and Csde1 protein. To do this, I compared mRNA localization using *in situ* hybridization (ISH) to protein immunofluorescent staining (IF) (Figure 11).

At E15.5 when protein expression was still low, Csde1 mRNA was seen in the VZ and CP (Figure 11A). This contrasted with the protein expression found primarily in the CP, with little expression in the VZ. Thus, it appeared that at E15.5 translation of Csde1 was repressed in the VZ. By E18.5 mRNA expression was found throughout the cortex, with higher expression in the upper layers of the CP and in the VZ (Figure 11B). However, protein distribution was restricted to the upper CP and shows little expression in the VZ. These results suggest that Csde1 expression may be regulated at both transcriptional and post-transcriptional levels in the E18.5 cortex.

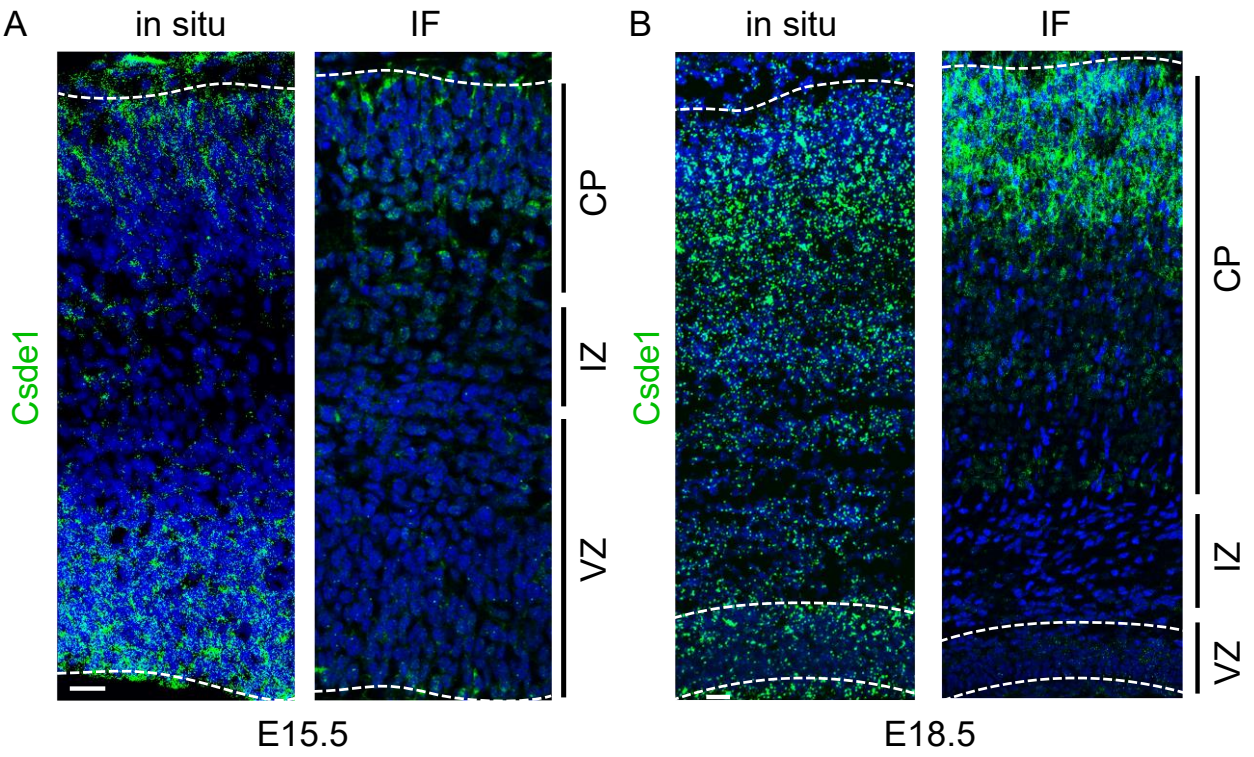

Figure 11. Csde1 mRNA and protein localization differ in the developing cortex. Fluorescent *in situ* hybridisation of Csde1 mRNA (left) compared to Csde1 protein expression by IHC (right) (A) E15.5 cortex shows mRNA expression in the VZ and cortical plate (left) while protein expression is restricted in the cortical plate (right). (B) E18.5 cortex shows mRNA expression through the cortex (left), while Csde1 protein (right) is restricted to the apical surface of the cortical plate. Cells counterstained with Hoechst. Cortical region denoted by dotted line. Scale bar = 30µM.

4.3 Summary

Csde1 is expressed in NPCs and neurons *in vitro*, localizing to RNA granules consistent with Csde1's role in RNA metabolism. In the developing cortex, Csde1 protein expression is restricted to the newborn upper layer neurons in contrast to ubiquitous expression of Csde1 mRNA throughout the cortex. This restricted localization, combined with the difference in

overall mRNA and protein levels, suggests that Csde1 protein expression is carefully regulated during the developmental periods in the cortex.

Patterned gene expression is an essential level of control in development. Restricted expression of mRNA and protein establish boundaries; these boundaries are used to instruct overall patterning and cell differentiation during development (Greig et al., 2013). The spatial restriction of Csde1 indicates it may be active in establishing or maintaining this developmental boundary during corticogenesis. The exclusion of Csde1 expression in the VZ may be purposeful if Csde1 affects self-renewal and differentiation of NPCs. Particularly of interest, the variable expression of Csde1 in layer V (Figure 10) indicates it may be active in the restriction of deep layer neuron generation or the specification of upper layer neuron generation.

Chapter 5: Csde1 regulates NPC self-renewal and differentiation

Given that Csde1 is expressed in NPCs, I wanted to determine whether Csde1 regulates NPC development. Previous work shows that Csde1 prevents the differentiation of hESCs to neuroepithelium, but it is not known how Csde1 affects NPC differentiation (Ju Lee et al., 2017).

5.1 Csde1 enhances neuron differentiation from NPCs

To examine the role of Csde1 on NPC differentiation, I took a loss- and gain-of-function approaches using short-hairpin RNAs (shRNAs) and overexpression, respectively. I first validated the efficiency of Csde1 shRNAs by transfection of human embryonic kidney cell line (HEK293) with a plasmid expressing mouse Csde1 (pEGFP-Csde1) together with control or Csde1 shRNAs. 48 hours after transfection, Western blot analysis showed that while the protein level of EGFP-Csde1 was unaffected by control shRNA (shLuciferase), its level was significantly reduced by Csde1 shRNA-1 (shR1) (Figure 12A). This indicates that Csde1 shR1 can effectively knock down Csde1 expression .

On the other hand, we overexpressed Csde1 by transfecting cultured cells with an expression plasmid that contains mouse Csde1 with a DDK tag under the control of the CMV promoter (Csde1-DDK) (Figure 12B). Western blot analysis of transfected HEK293 cells with an anti-Csde1 antibody shows that the expression of exogeneous Csde1-DDK led to a two-fold increase in Csde1 expression, consistent with a robust signal detected by an anti-DDK antibody (Figure 12B). To further confirm the expression of Csde1-DDK in cultured NPCs, I transfected the plasmid to E12.5 primary NPC cultures together with a plasmid expressing EGFP (pCAGIG) as a tracker to detect transfected cells. Immunostaining of cultured NPCs three days after

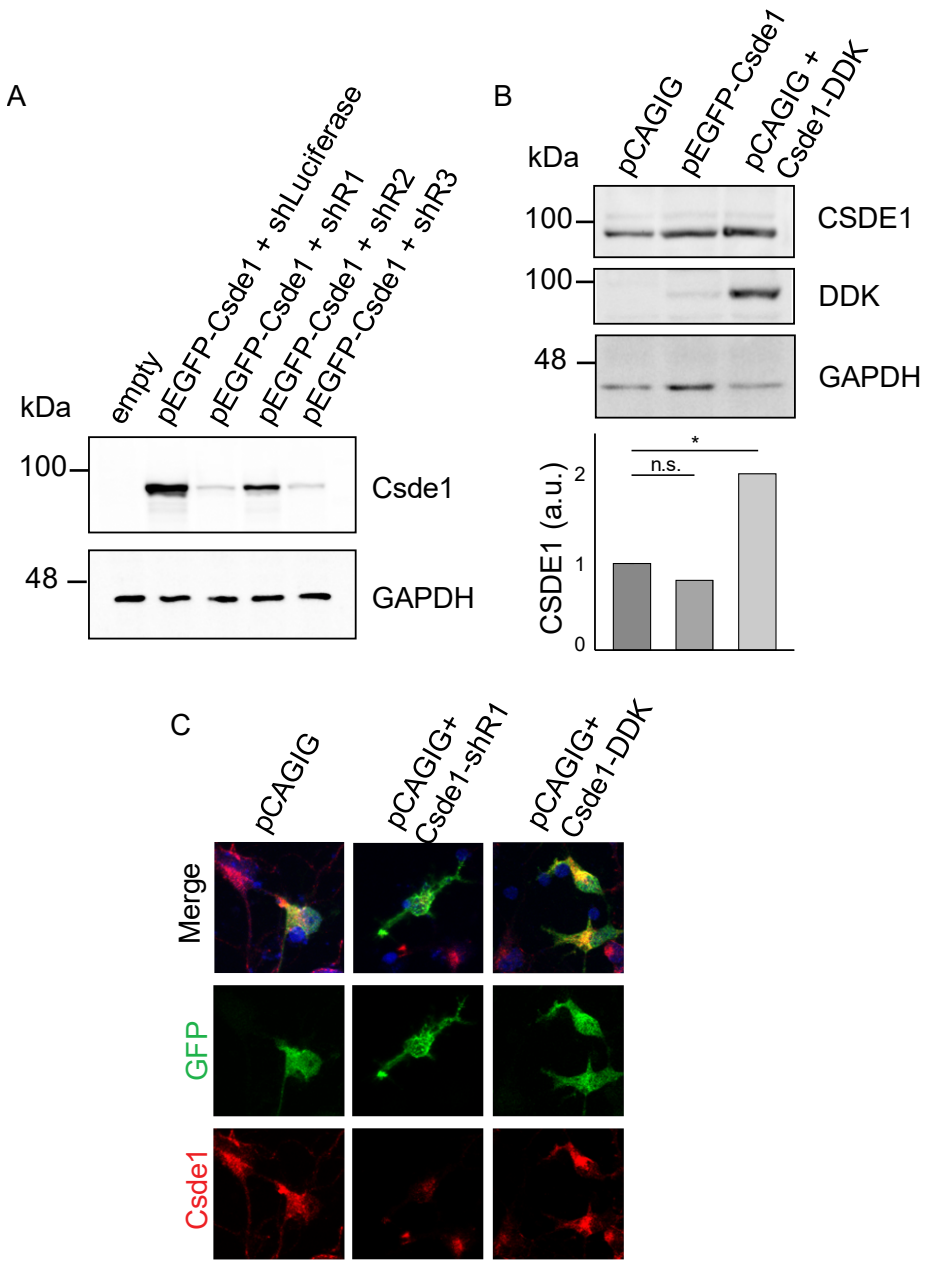

Figure 12. Validation of Csde1 transfection vectors. (A) Western blot of pEGFP-Csde1 transfected HEK293. Co-transfection of control shRNA construct against luciferase (shLuciferase) or target shRNA against Csde1 (shR1-3). (B)Western blot and analysis of Csde1 overexpression by pEGFP-Csde1 and Csde1-DDK construct in HEK293 cells. Fold change quantified by normalization to GAPDH loading control. n=3 *p<0.05. Students t-test. (C) Representative staining of primary cultured cortical NPCs. DIV3.

transfection showed a robustly higher signal in cells transfected with Csde1-DDK while Csde1 expression was reduced upon the expression of Csde1 shR1 (Figure 12C). Together, these data indicate the effectiveness of the shRNA and overexpression plasmids for further functional assessment of Csde1 in cultured NPCs.

Using the validated Csde1-DDK and Csde1shR1 constructs, I next examined the effect of Csde1 overexpression and knockdown on NPCs. Primary NPCs from E12.5 embryos were cultured for 4 hours prior to co-transfection of pCAGIG and either Csde1-DDK or Csde1shR1. Using immunofluorescent staining of NPC marker Sox2, and neuron marker β-III tubulin, construct transfected GFP tagged cells were counted to determine the proportion of NPCs and neurons after 3 days in culture (Figure 13). In cells transfected with a control vector, 44% of cells were Sox2+ NPCs, while 53% of cells were β-III tubulin+ neurons. This provides a baseline measure of stem cell differentiation in cultured NPCs. Knockdown of Csde1 significantly increased the NPC population ($p<0.01$) to 64%, reducing the neuron population ($p=0.02$) to 32%. Conversely, overexpression of Csde1 significantly reduced the NPC population ($p=0.02$) to 25% and increased the proportion of neurons to 73% ($p=0.04$). Together, knockdown and overexpression of Csde1 indicate that Csde1 promotes neuronal differentiation over self-renewal of NPCs *in vitro.*

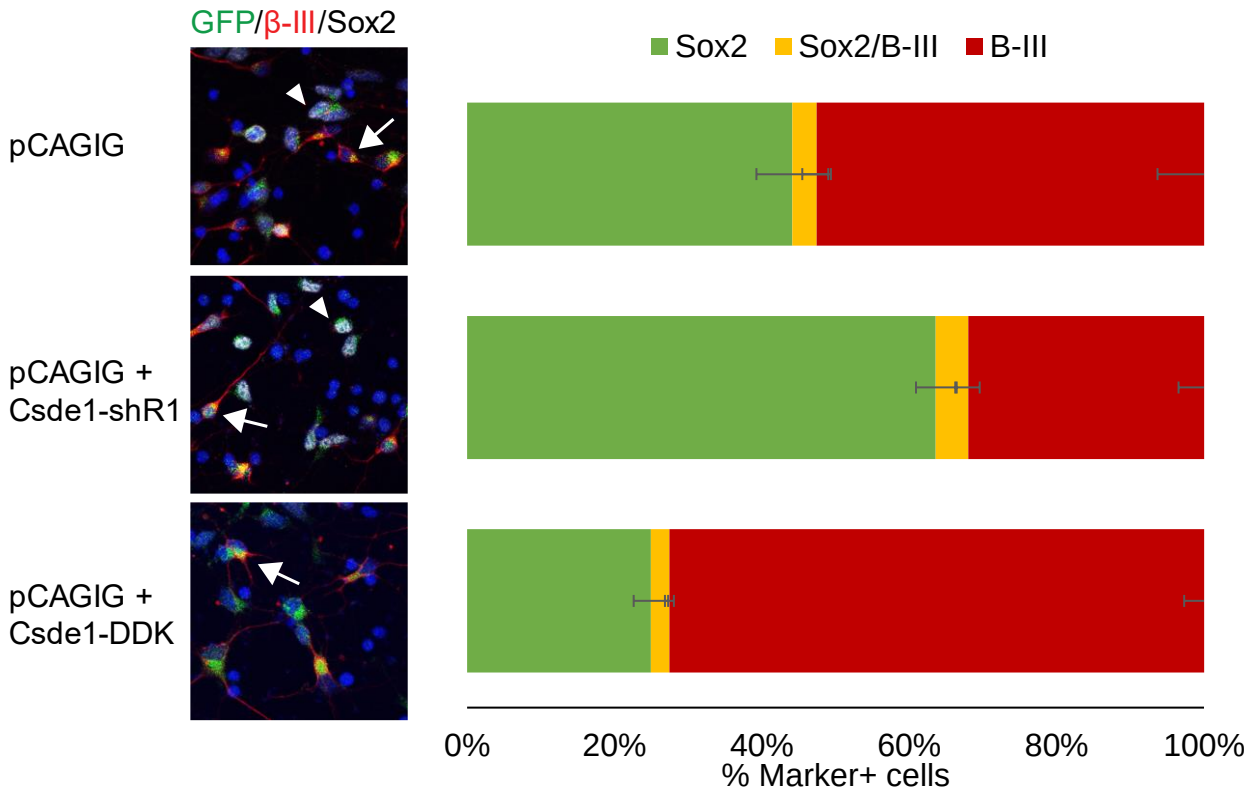

Figure 13. Csde1 levels affect differentiation of primary NPCs *in vitro*. Transfection of primary NPC cultures (left) show Sox2+ cells (arrowhead) and β-III+ cells (arrow). pCAGIG used as tracker for NPC transfection. Control pCAGIG transfection, 44% Sox2+, 3% double positive, 53% β-III+ (n=471). shR1 transfection, 64% Sox2+, 4% double positive, 32% β-III+ (n=682). Csde1-DDK transfection, 25% Sox2+, 3% double positive, 72% β-III+ (n=806). Cells counterstained with Hoechst (blue). n=total cells counted during 4 trials. Statistical analysis done using students t-test (n=4).

5.2 Heterozygous knockout of Csde1 reduces Csde1 expression

Since expression of Csde1 affects NPC differentiation *in vitro*, I next wanted to determine if Csde1 influences neurogenesis *in vivo*. To this end, I used a transgenic mouse model, where Csde1 has an insertion of LacZ ($Csde1^{tm1e/+}$) gene trap (Figure 14A). This resulted in reduced protein expression of Csde1, measured by western blot (Figure 14A-C). This model is heterozygous for Csde1 disruption, as homozygous loss of Csde1 is embryonic lethal, and homozygous progeny are never found (Boussadia et al., 1997). This heterozygous Csde1 knockout mouse will be referred to as $Csde1^{KO/+}$.

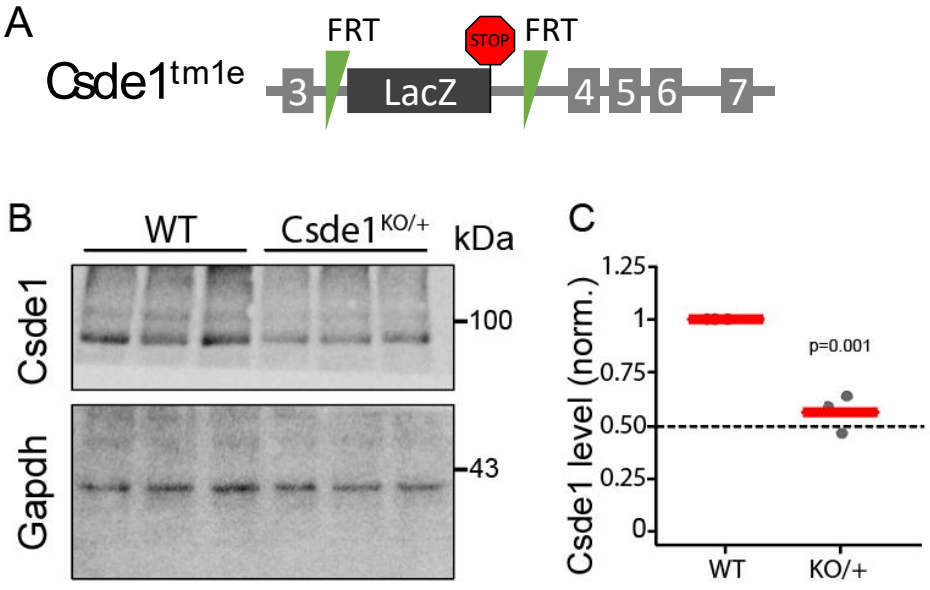

Figure 14. $Csde1^{KO/+}$ heterozygotes show reduced Csde1 expression. (A) diagram of tm1e gene trap allele. Csde1 exon 3 is spliced to LacZ with a stop codon, preventing full length Csde1 expression. (B-C) Western blot analysis of Csde1 protein in the cortex at E18.5, normalized to Gapdh. n=3, represented as points on scatter plot. Student t-test.

It is using this heterozygous Csde1$^{KO/+}$ model that I will examine the effects of Csde1 *in vivo.* To this end, I examined neurogenesis in the cortex at embryonic day 18.5 (E18.5), as well as post-natal day 7 (P7). At E18.5 neurogenesis is completed, and RGs begin gliogenesis. During this time, neurons are migrating to their final locations. By P7, neuronal migration is mostly completed, providing a view of gross cortical patterning.

5.3 Heterozygous Csde1 knockout does not affect neurogenesis *in vivo*

To assess neurogenesis *in vivo* I examined the overall size of the cortical plate as well as total cell number within the cortex using coronal sections. In addition to size and cell number, I also examined the distribution of cells within the cortical plate. These parameters allow us to determine if there are gross morphological differences in response to loss of Csde1, as well as subtle changes in neuron distribution.

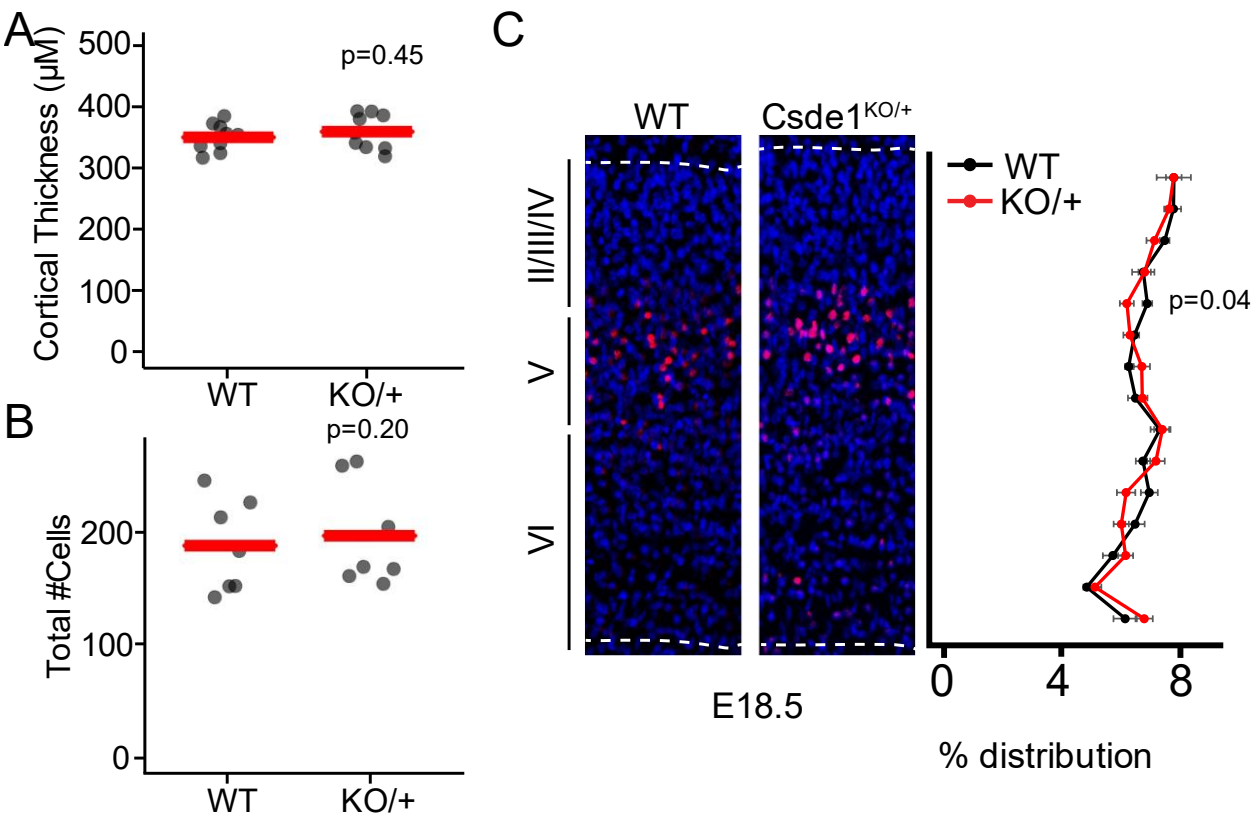

Figure 15. Csde1 heterozygotes have normal cortical plate size and density at E18.5.

(A) Overall depth as measured from the basal surface of layer VI to the top surface of layer II (B) Total cell number of sampled cortical column counted using Hoechst staining. (C) Representative image of cortical plate, ctip2 (red) used as layer reference. Average neuron distribution (cells in bin/total) shown (right). n=7. Students t-test.

At E18.5 overall cortical plate size was unaffected in Csde1 mutant mice (p=0.45), cell number counted by nuclear Hoechst staining was also unaffected (p=0.20) (Figure 15A,B). This indicated that at E18.5 there is not an extreme defect cortical expansion or neurogenesis due to reduction of Csde1 expression. Although the overall size and cell numbers remained constant, Csde1$^{KO/+}$ resulted in altered neuron distribution within the cortical plate. In the E18.5 cortex,

Csde1 heterozygotes showed slightly reduced distribution of neurons in layer IV (p=0.04), and this is compensated by slight increases in distribution through layer V (Figure 15C).

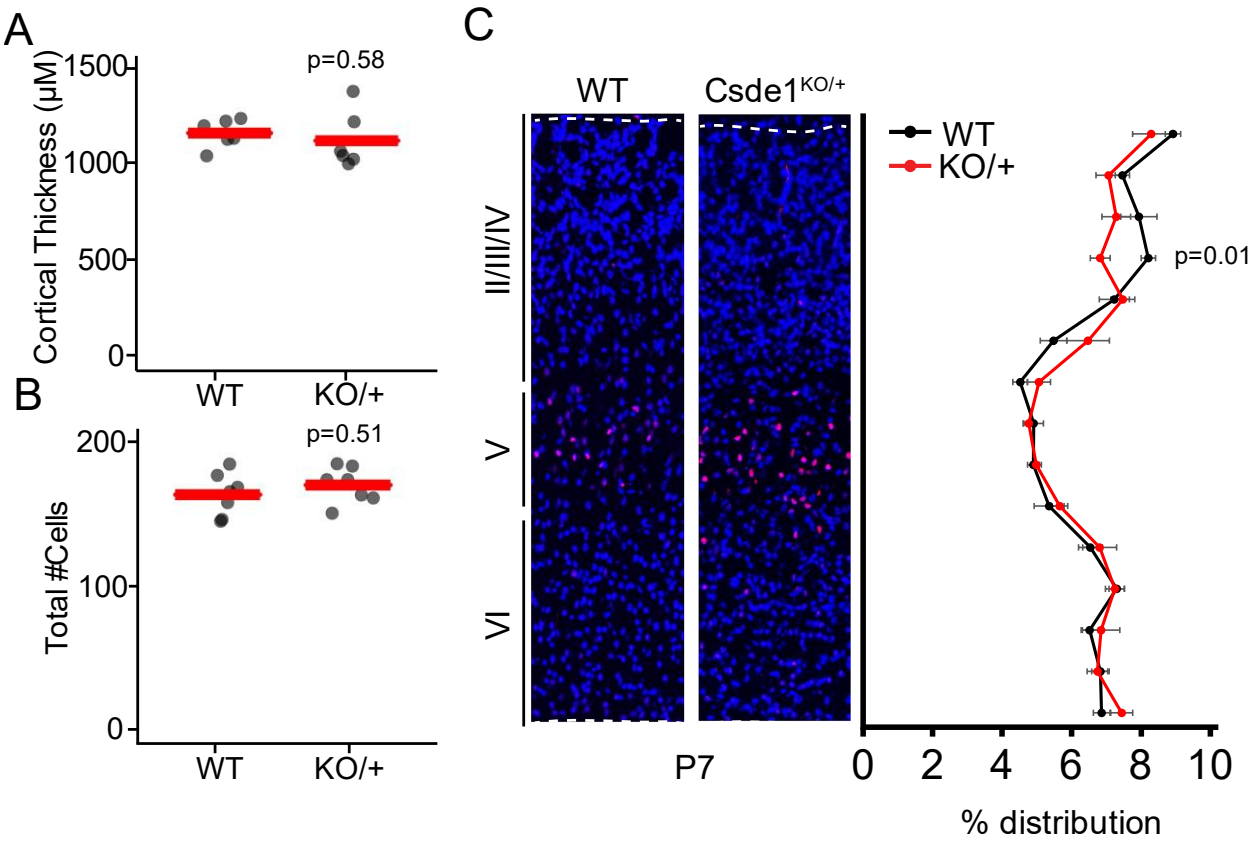

Figure 16. Csde1 heterozygotes have normal cortical plate size and density at P7. Coronal section of post-natal cortex. (A) Overall depth as measured from the basal surface of layer VI to the top surface of layer II. (B) Total cell number of sampled cortical column counted using Hoechst staining. (C) Representative image of cortical plate, ctip2 (red) used as layer reference. Average neuron distribution (cells in bin/total) shown (right). n=7. Students t-test.

By P7, overall cortical size and neuron numbers remain consistent between Csde1$^{KO/+}$ and control cortices (p=0.58, p=0.51) (Figure 16A,B). During cortical expansion, between E18.5 and P7, changes seen in neuron distribution become more pronounced. At P7, reduced distribution was observed in layers II/III (p=0.01) while increased distribution is spread into lower layer III and IV (Figure 16C), determined by layer markers Satb2 and Ctip2 (see Chapter 6) obscuring the boundary between the upper and mid layers of the CP. This is consistent with expansion of the altered distribution observed at E18.5. This indicates that although Csde1 loss may have little impact on neurogenesis, it may affect the migration and distribution of neurons in the cortex.

5.4 Summary

Csde1 is pro-neurogenic in primary cultured NPCs: knockdown of Csde1 restricts NPC differentiation while Csde1 overexpression promotes NPC differentiation. Although alterations in the level of Csde1 are sufficient to affect neurogenesis *in vitro,* heterozygous knockout of Csde1 seems to have little effect on overall cell numbers *in vivo.* Cortical size and cell numbers are conserved in the Csde1$^{KO/+}$ cortex. It is possible that Csde1 protein expression is already low in the VZ during the proliferative period (Figure 10), thus heterozygous loss of Csde1 in dividing cells may have little effect on overall neurogenic potential in the cortex. It may also reflect the limitation of the used experimental approach. Although overall cell number is persevered in Csde1$^{KO/+}$, neuron distribution is altered, indicating that there may be defects in neuron maturation and migration. However, due to the small sample size and technical limitations, further analysis is needed to confirm whether Csde1 regulates neurogenesis and alters the distribution of neuronal subtypes *in vivo*.

Chapter 6: Csde1 regulates neuronal subtype specification

In addition to neurogenesis, specification of neurons plays a critical role in corticogenesis. Producing the correct number of neurons is insufficient for cortical function; neurons must mature and migrate to their assigned laminar position. Defects in neuron specification affect patterning, lamination and function of cortical neurons (Greig et al., 2013; Molyneaux et al., 2007). I show that the distribution of cortical neurons is affected in $Csde1^{KO/+}$ mice.

6.1 Knockdown of Csde1 restricts upper layer neuron generation

Using transfection of primary NPCs, neuronal subtype was examined *in vitro* after 3 days in culture. This was done using pCAGIG co-transfection with either Csde1-DDK or Csde1shR1, as described previously. Given the changes in neuron distribution in layers II-V, I focused on the distribution of upper layer Satb2+ neurons and layer V Ctip2+ neurons. Additionally, Satb2+ neurons are the primary neuron type generated by NPCs at E12.5 when cells were harvested for culture.

Transfection of control vector shows that 81% of GFP+ co-transfected neurons were Satb2+, while only 10% expressed Ctip2, this is expected given the use of E12.5 NPCs, which generate Satb2+ cells. Knockdown of Csde1 using Csde1shR1 greatly reduced the Satb2+ neurons population to only 24% ($p<0.01$) and increased the Ctip2+ population to 66% ($p>0.01$) and did not significantly affect the co-expression of neuronal subtype markers. Overexpression of Csde1 did not significantly affect the Satb2+ or Ctip2+ populations (76% and 10% respectively) (Figure 17).

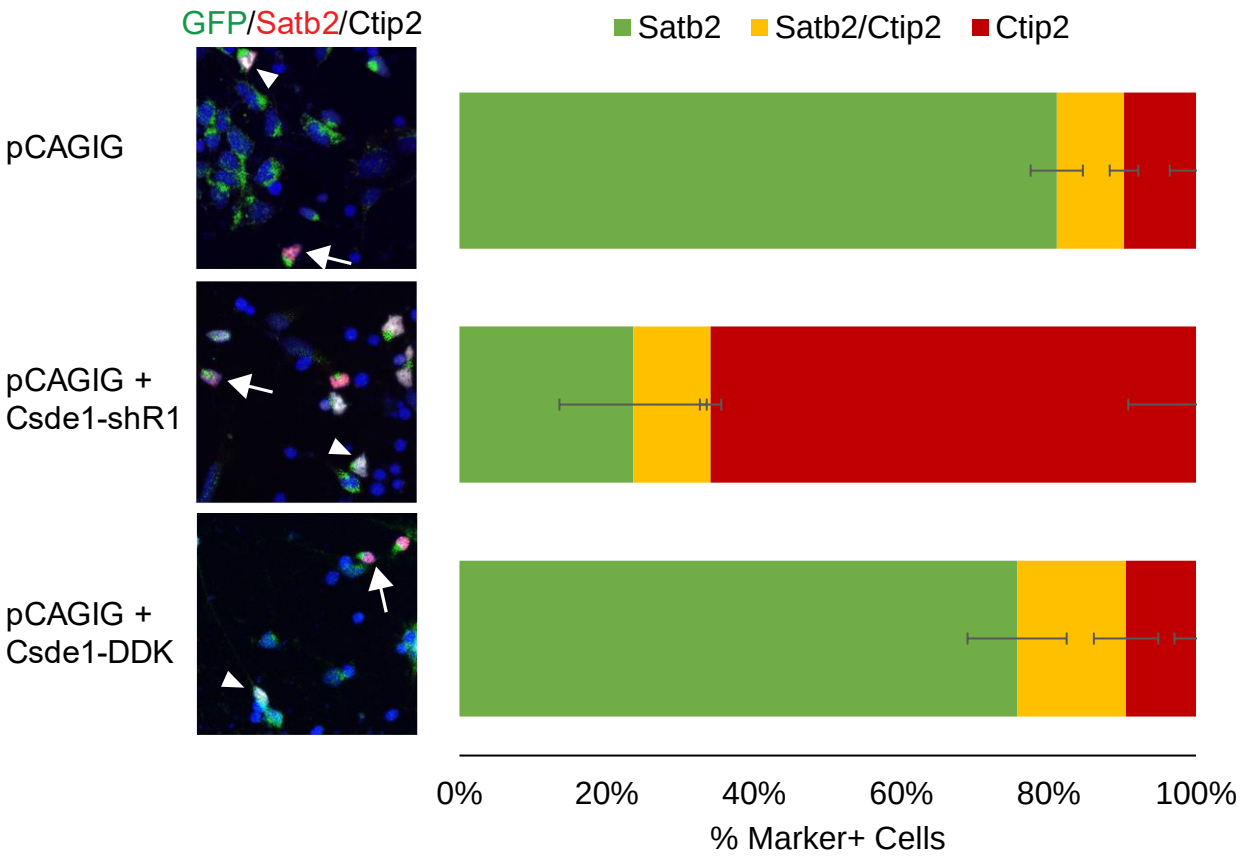

Figure 17. Csde1 levels alter deep and upper layer neuron specification *in vitro*.

Transfection of primary NPC cultures (left) show Ctip2+ cells (arrowhead) and Satb2+ cells (arrow). pCAGIG used as tracker for NPC transfection. Control pCAGIG transfection, 81% Satb2+, 9% double positive, 10% Ctip2+ (n=550). shR1 transfection, 24% Satb2+, 10% double positive, 66% Ctip2+ (n=394). Csde1-DDK transfection 76% Satb2+, 15% double positive, 9% Ctip2+ (n=532). Counterstained with Hoechst (blue). n=total cells counted during 4 trials. Statistical analysis done using students t-test (n=4).

The increase of layer V Ctip2+ neurons in the Csde1 knockdown condition, indicates that Csde1 may be responsible for either promotion of upper layer specification or restriction of layer

V specification. Additionally, reduced neurogenesis in Csde1shR1 cultures may prevent expansion of E12.5 NPCs, which produce Satb2+ neurons, leading to an increased proportion of Ctip2+ neurons.

6.2 Layer identity within the cortex is altered in Csde1$^{KO/+}$

Alteration of neuronal subtype *in vitro* indicates that Csde1 regulates neuronal subtype specification. To examine further, and to confirm the phenotype observed in primary culture, I used the Csde1$^{KO/+}$ mouse. In coronal sections, subtype markers can be used to distinguish lamination of the cortical plate. To this end I used 3 subtype markers: Tbr1 marking layer VI neurons, Ctip2 marking layer V neurons and Satb2 marking upper layer II/III neurons. Using this method, I examined the total number of each neuronal subtype, as well as their distribution within the cortical plate at E18.5 and P7.

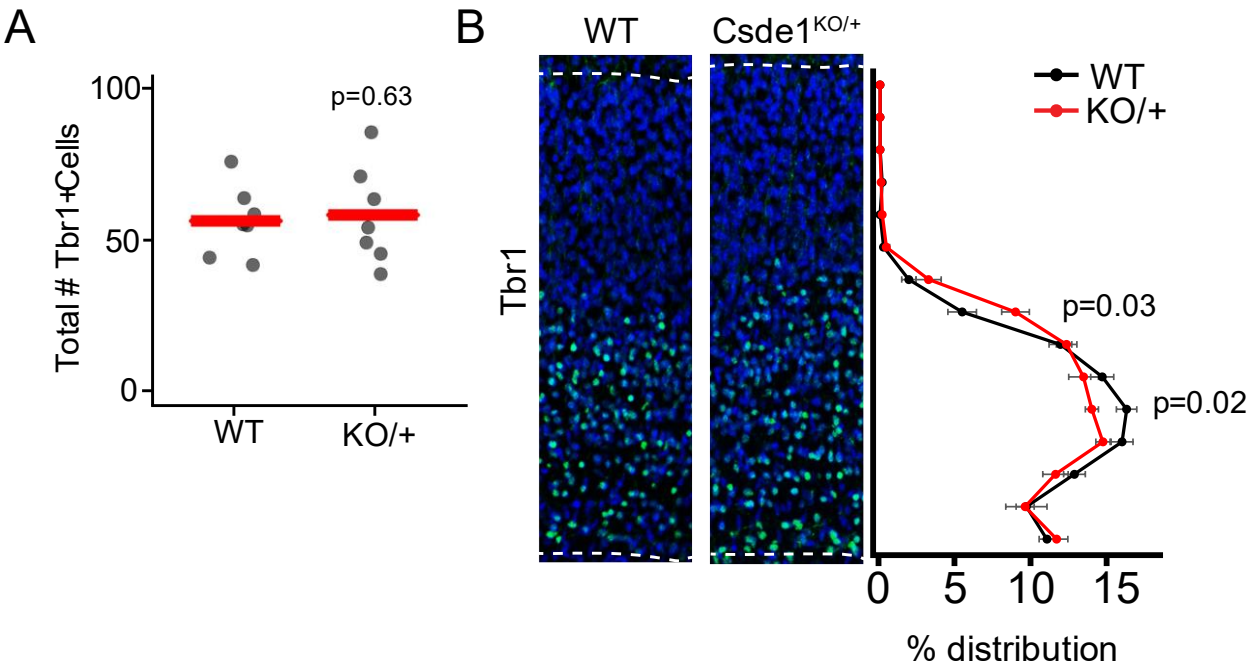

Figure 18. Distribution of deep layer neurons is altered in Csde1$^{KO/+}$ mice at E18.5. (A) total Tbr1+ cells in sampled column. Each dot represents one mouse. (B) Representative image of cortical plate. Tbr1+ neurons (green). Average distribution of Tbr1+ cells/total cells (right). Counterstained with Hoechst (blue). n=7. Student t-test.

Csde1 heterozygotes at E18.5 did not show significant differences in the total number of each neuronal subtype (Figure 18,19,20). Although the total number of each subtype was consistent, the distribution across the CP was shifted in each case. Deep layer Tbr1+ neurons were found towards the pial surface (p=0.03) resulting in reduction in the center of layer VI (p=0.02) (Figure 18). This shift towards the pial surface was also observed in layer V (Figure 19), where Ctip2+ neurons extended into layer II/III (p=0.02).

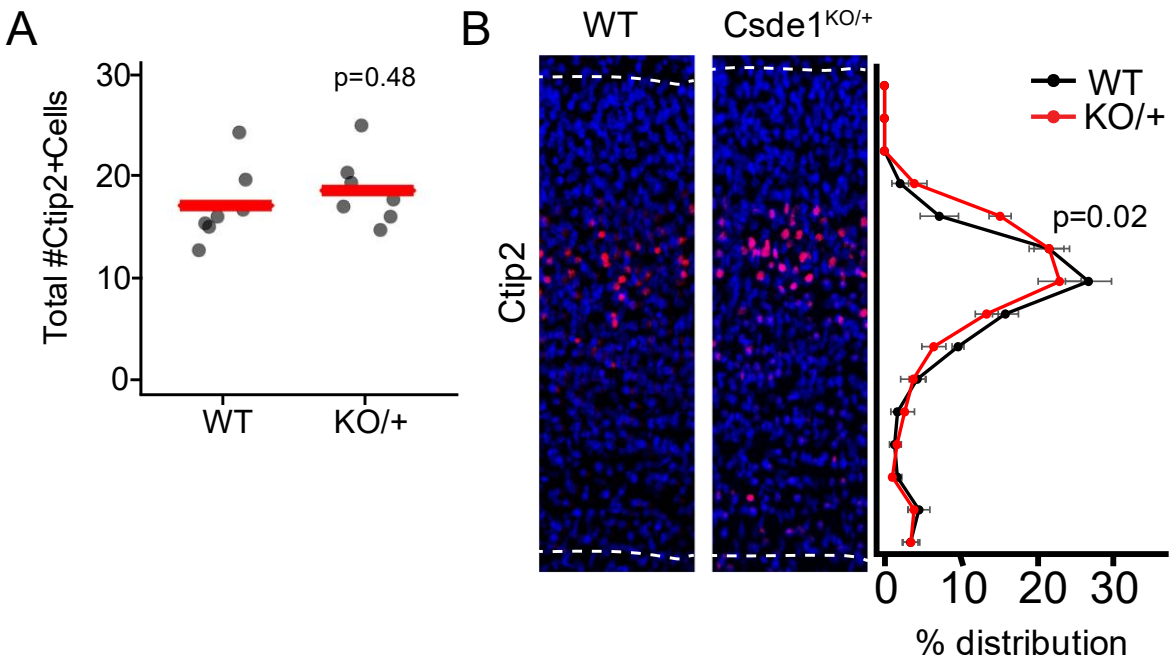

Figure 19. Distribution of layer V neurons in altered in Csde1$^{KO/+}$ mice at E18.5. (A) total Ctip2+ cells in sampled column. Each dot represents one mouse. (B) Representative image of cortical plate. Ctip2+ neurons (red). Average distribution of Ctip2+ cells/total cells (right). Counterstained with Hoechst (blue). n=7. Student t-test.

Given the extension of the deep layers towards the pial surface I expected compression of layers II/III. However, I observed extension of Satb2+ neurons throughout the CP (Figure 20). Distribution was reduced in the upper compartments (p=0.04) and increased in layer IV-V (p=0.04). This obscured the upper layer boundary in the CP. At E18.5 migration of upper layer neurons is still incomplete in the CP, and this expansion may be a result of delayed migration or improper assignment of fully migrated neurons. To further distinguish the cause of upper layer expansion, the CP at P7 was examined.

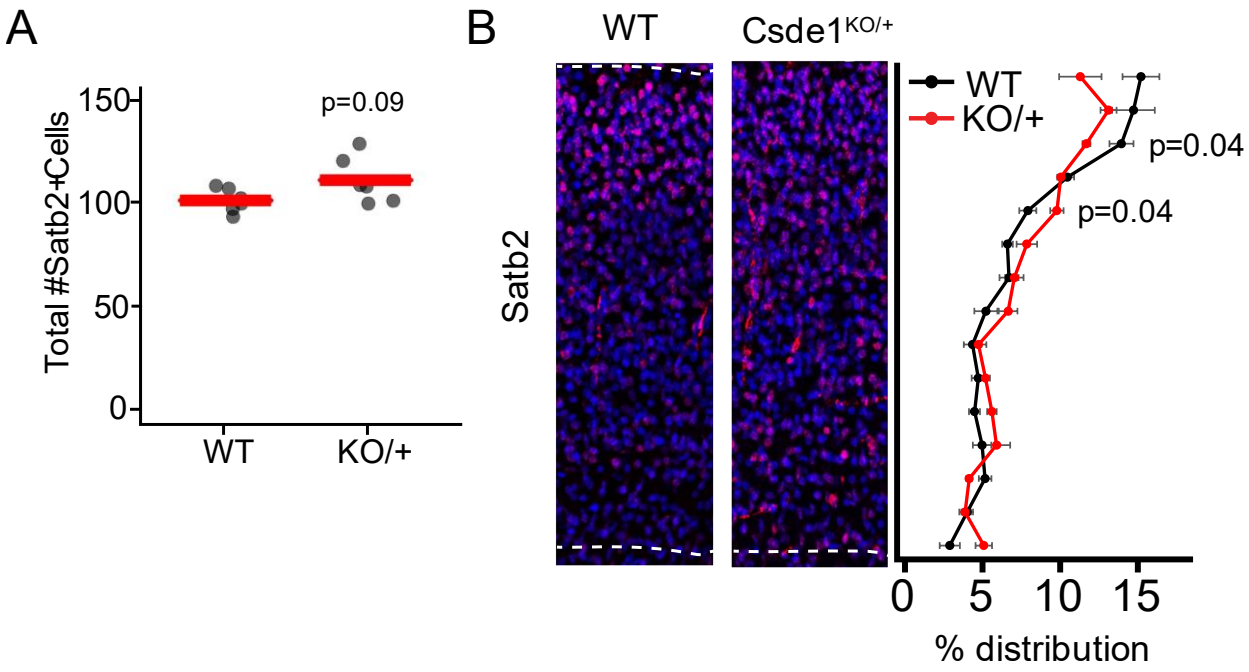

Figure 20. Distribution of upper layer neurons in altered in Csde1$^{KO/+}$ mice at E18.5. (A) total Satb2+ cells in sampled column. Each dot represents one mouse. (B) Representative image of cortical plate. Satb2+ neurons (red). Average distribution of Satb2+ cells/total cells (right). Counterstained with Hoechst (blue). n=7. Student t-test.

This disturbance of neuronal subtype at E18.5 was moderate, and determining if it persisted post-natally was important to determine if Csde1$^{KO/+}$ is sufficient to cause long term alteration of the CP.

At P7, total number of each subtype increased compared to wildtype controls. In the region assessed, layer VI Tbr1+ cells increased from an average of 127 cells to 173 cells (p=0.03) (Figure 21), layer V Ctip2+ cells increased from an average of 31 cells to 45 cells (p=0.04) (Figure 22) while upper layer Satb2+ cells increased from 274 to 340 (p>0.01) (Figure

23). This suggests that subtype specification is altered in the Csde1$^{KO/+}$ environment, as the total number of neurons was unaffected at P7.

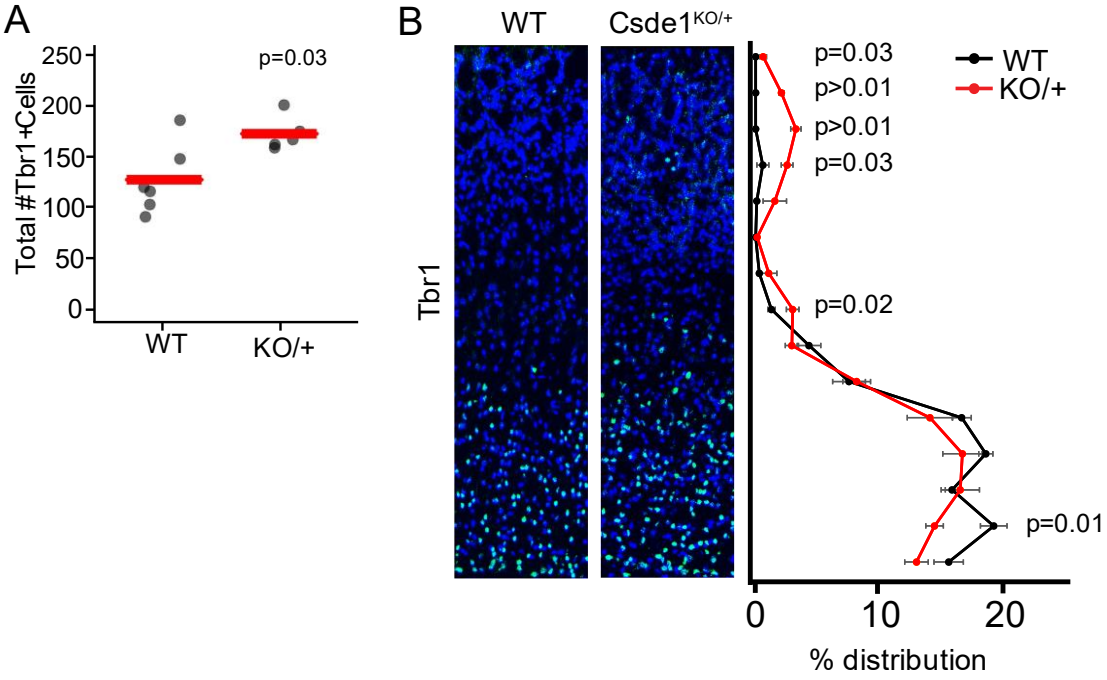

Figure 21. Deep layer neuron number and distribution is altered in Csde1$^{KO/+}$ cortices at P7. (A) total Tbr1+ cells in sampled column. Each dot represents one mouse. (B) Representative image of cortical plate. Tbr1+ neurons (green). Average distribution of Tbr1+ cells/total cells (right). Counterstained with Hoechst (blue). n=6. Student t-test.

The increase in the total number of each cell type also affected the distribution of neuronal subtypes within the CP. Tbr1+ neurons were seen extending towards the pial surface (p=0.02) and were found ectopically in layers II/III (p<0.01) (Figure 21). Ctip2+ neurons extended basally, into layer VI (p=0.03) reducing their proportion in layer V (p=0.04) (Figure 22). 0

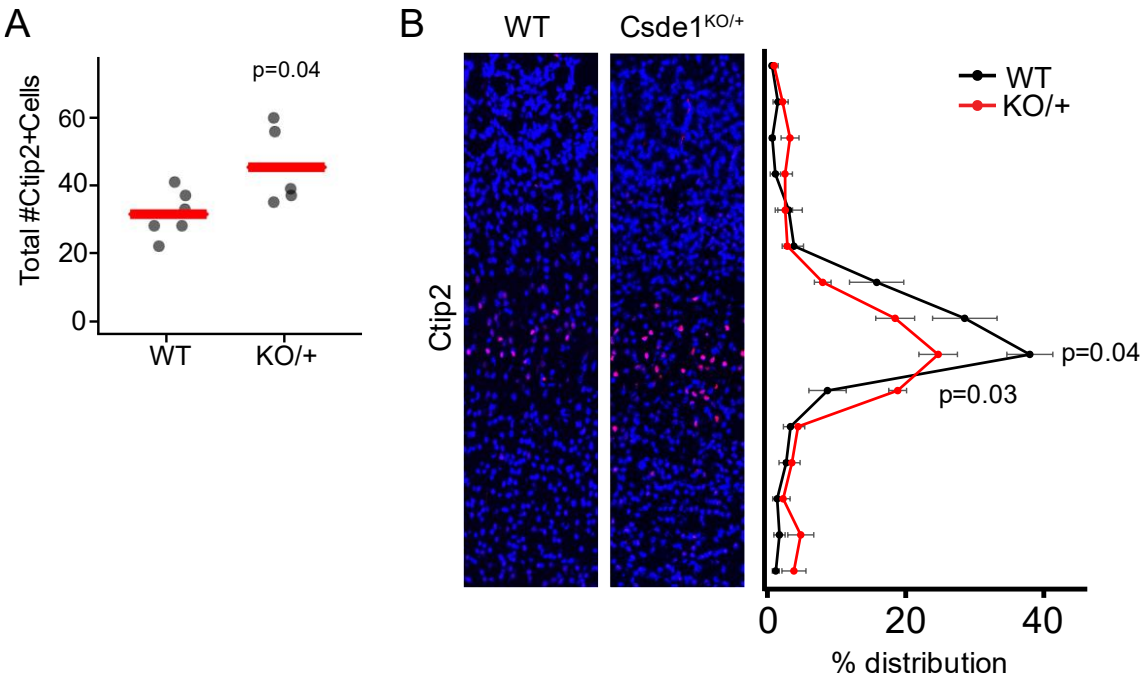

Figure 22. Layer V neuron number and distribution is altered in Csde1$^{KO/+}$ cortices at P7. (A) total Ctip2+ cells in sampled column. Each dot represents one mouse. (B) Representative image of cortical plate. Ctip2+ neurons (red). Average distribution of Ctip2+ cells/total cells (right). Counterstained with Hoechst (blue). n=6. Student t-test.

As with E18.5, Satb2+ neurons were found extending towards the basal surface with increased distribution in layer IV (p=0.04) reducing distribution in layer II. (p=0.02) (Figure 23). This persistent phenotype at P7 indicates that the expansion of Satb2+ cells at E18.5 was not due to retarded migration, as these Satb2+ cells never reach their destination compared to wildtype controls. This means that either subtype specification of Satb2+ neurons is overtaking layer V and VI neurons, or migration is halted during development.

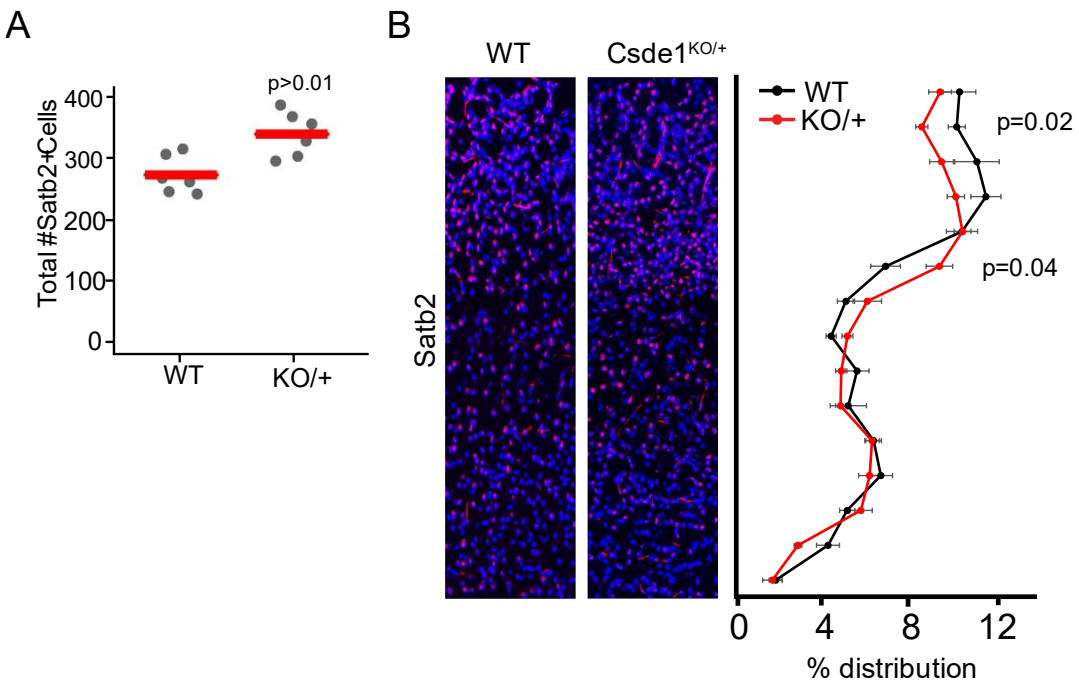

Figure 23. Upper layer neuron number and distribution is altered in Csde1$^{KO/+}$ cortices at P7. (A) total Satb2+ cells in sampled column. Each dot represents one mouse. (B) Representative image of cortical plate. Satb2+ neurons (red). Average distribution of Satb2+ cells/total cells (right). Counterstained with Hoechst (blue). n=6. Student t-test.

6.3 Subtype identity is altered in Csde1$^{KO/+}$

Abnormal subtype distribution at E18.5 and P7 may contribute to other phenotypic changes in Csde1$^{KO/+}$ mice. The increase in all neuronal subtypes at P7 indicates that subtype specification is disrupted. As a result, I looked at co-expression of neuronal subtype specifiers to determine if subtype assignment was completed. Increased co-expression of mutually exclusive subtype markers indicates disruptions in cell fate assignment.

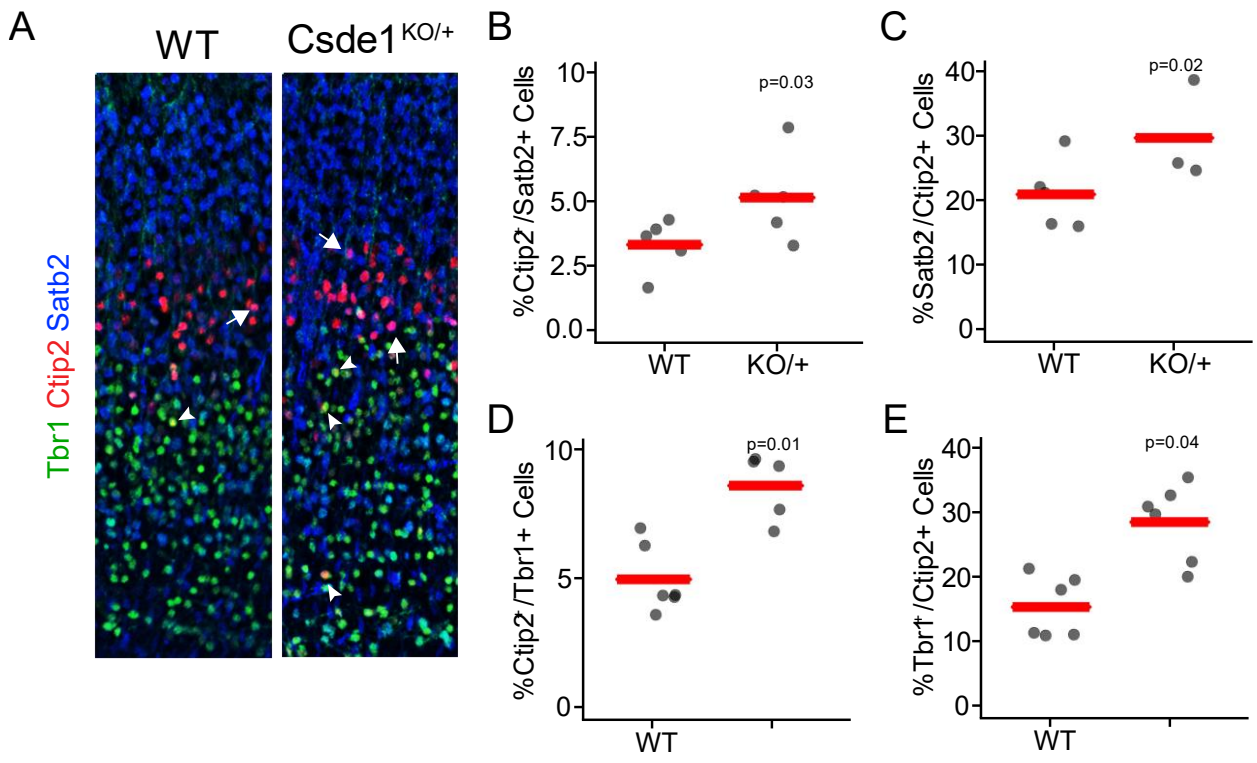

Figure 24. Csde1$^{KO/+}$ cortices have more neurons with mixed subtype identity at E18.5. (A) coronal section of E18.5 cortex. Tbr1/Ctip2 double positive cells (arrowheads) and Ctip2/Satb2 double positive cells (arrows). (B) Percent Ctip2+/Satb2+ cells per total Satb2+ cells. (C) Percent Ctip2+/Satb2+ cells per total Ctip2+ cells (D) Percent Ctip2+/Tbr1+ cells per total Tbr1+ cells. (E) Percent Ctip2+/Tbr1+ cells per total Ctip2+ cells. Each dot represents one mouse. n=6. Students t-test.

At E18.5, Csde1$^{KO/+}$ mice showed a greater proportion of double positive cells (Figure 24, 25). The proportion of Ctip2+/Satb2+ cells increased from 3% to 5% (p=0.03), while the inverse (Satb2+/Ctip2+) increased from 21% to 35% (p=0.02) (Figure 24B,C). The reciprocal increase indicates that the subtype assignment is incomplete, as both markers are abnormally expressed in the opposite population. The same is true for Ctip2+ cells/Tbr1+ cells which increased from 5%

to 10% (p=0.01), with the inverse (Tbr1+/Ctip2+) increased from 15% to 29% (p=0.04) (Figure 24D,E).

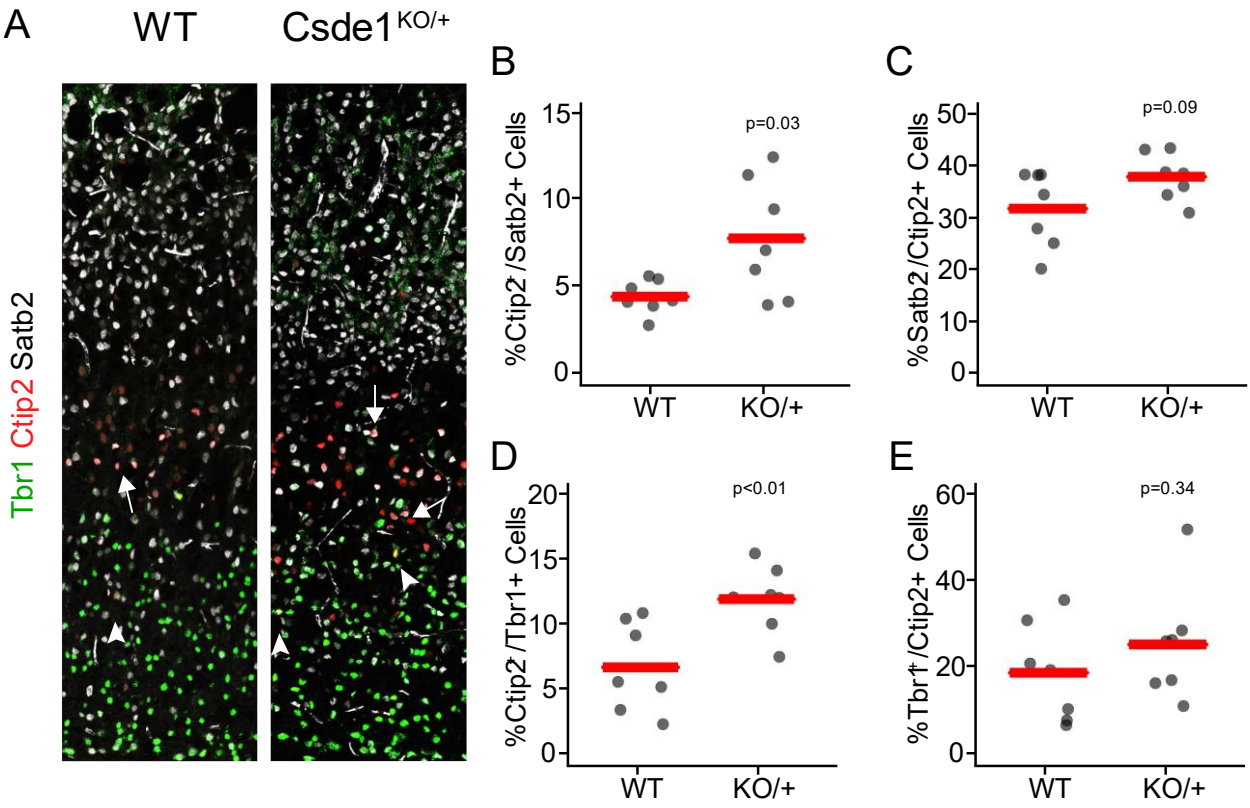

Figure 25. Csde1$^{KO/+}$ cortices have more neurons with mixed subtype identity at P7. (A) coronal section of P7 cortex. Tbr1/Ctip2 double positive cells (arrowheads) and Ctip2/Satb2 double positive cells (arrows). (B) Percent Ctip2+/Satb2+ cells per total Satb2+ cells. (C) Percent Ctip2+/Satb2+ cells per total Ctip2+ cells (D) Percent Ctip2+/Tbr1+ cells per total Tbr1+ cells. (E) Percent Ctip2+/Tbr1+ cells per total Ctip2+ cells. Each dot represents one mouse. n=7. Students t-test.

By P7, there is still increased co-expression of subtype markers (Figure 25A), however the relationship is no longer reciprocal. Ctip2+/Satb2+ increased from 4% to 8% (p=0.03) while the inverse did not show a significant increase (32% to 37%, p=0.09) (Figure 25B,C). Ctip2+/Tbr1+ increased from 7% to 12% (p>0.01) in $Csde1^{KO/+}$ while Tbr1+/Ctip2+ did not increase significantly (19% to 25%, p=0.34) (Figure 25D,E). Loss of this inverse relationship indicates that Ctip2+ expression is increased in neurons which would ordinarily be Tbr1+ or Satb2+. This ectopic Ctip2+ expression could be stalling subtype specification leading to increased co-expression and increased number of all neuronal subtypes in the CP.

6.4 Csde1 is haploinsufficient for complete subtype assignment in the murine cortex

Csde1 heterozygotes show abnormal subtype distribution and increased co-expression of subtype specifiers. This indicates that although heterozygous loss of Csde1 is sufficient for normal neurogenesis, it is insufficient for neuronal subtype assignment. $Csde1^{KO/+}$ mice show abnormal neuronal distribution, and abnormal subtype specification. This is seen at E18.5, as well as P7. This includes increased numbers of neuronal subtypes at P7, and increased overlap of these subtypes. This indicates that subtype specification is compromised in Csde1 heterozygotes.

6.5 Summary

In primary NPC culture, knockdown of Csde1 increases layer V Ctip2+ neurons, reducing upper layer Satb2+ neurons. This indicates that Csde1 either restricts upper layer differentiation or promotes layer V generation. Overexpression of Csde1 does not significantly impact subtype. This could be because Csde1 is self-regulatory, binding its own transcript, this may prevent its

activity in neurons. In addition, Csde1 is highly expressed physiologically in neurons, and overexpression may have been insufficient to influence subtype.

At E18.5 neurogenesis and subtype numbers are unaffected by heterozygous knockout of Csde1, although subtype co-expression is increased. By P7, subtype assignment is altered, with overabundance of all neuronal subtypes. This also increases overall subtype overlap. However, at P7, Ctip2+ cells increase over their deep layer and upper layer competitors. It is worth mentioning that the immunostaining analysis was potentially influenced by the inconsistent background signals. While further studies are needed, my results indicate that Csde1 may be promoting Ctip2 layer V fate, altering the overall cortical landscape through the layer V transition.

Chapter 7: Discussion

Csde1 is a cytoplasmic RNA binding protein. Previous work has established that Csde1 affects neuronal connectivity and stem cell differentiation (Guo et al., 2019; Ju Lee et al., 2017). Here, I demonstrate that Csde1 affects NPC differentiation and neuronal subtype specification.

Csde1 is expressed during cortical development, increasing during the developmental window. This expression is regulated post-transcriptionally and shows patterned expression within the developing cortex. Csde1 mRNA is expressed across the developing cortex, while protein expression is restricted to the upper layers. This patterned expression indicates that Csde1 may be regulated at the translational level, and restriction of Csde1 expression could be essential for its role in development.

In this study I show that Csde1 alters NPC regulation *in vitro*, enhancing neuronal differentiation in primary NPCs. This is in contrast to previous work in hESCs, which shows that Csde1 restricts neuronal differentiation (Ju Lee et al., 2017). This previous work uses mRNA expression of NPC and neuronal markers to assess neuronal differentiation. They show that Csde1 knockdown increases neuronal marker expression, however, knockdown also increases NPC marker Pax6 expression (Ju Lee et al., 2017). As a result, it cannot be said that knockdown of Csde1 enhances only neuronal differentiation, and it may alter the NPC population as well. Further examination of the effect of Csde1 on neurogenesis is required.

Expression of Csde1 is controlled in the proliferative zone of the VZ at E15.5 and E18.5. At E15.5 there are some Csde1+ cells in the VZ, consistent with neurogenesis. While at E18.5, when neurogenesis is nearly complete, Csde1 expression is almost completely absent from the VZ. This restriction from the proliferative zones may be intentional, preventing acceleration of

neurogenesis and maintaining the stem cell population. As a result, reduced Csde1 expression in Csde1$^{KO/+}$ does not affect overall neuronal number in the cortical plate. It is important to note that neurogenesis in the cortex was not assessed directly in this study and follow up using lineage tracing of NPCs will be required to assess neurogenesis directly.

Csde1 is also observed in migrating cells at E15.5, suggesting that it may play a role in regulating the migration of immature neurons. The association of Csde1 with cellular migration is consistent with other studies, which indicate Csde1 is an essential migratory factor, and alters the expression of cellular adhesion proteins such as Cadherin2 (El Khouri et al., 2021; Kobayashi et al., 2013; Martinez-Useros et al., 2019). If Csde1 alters migration cortical neurons, it may also alter migration of inhibitory interneurons from the MGE and LGE. This would alter the excitatory/inhibitory balance within the cortex, affecting cortical function (Kwan et al., 2012; Lodato et al., 2011). Alterations in the excitatory/inhibitory balance in the cortex is associated with ASD and epilepsy (Bozzi et al., 2018). The effect of Csde1$^{KO/+}$ on migration was not addressed in this study.

During mid-cortical development at E15.5, Csde1 is expressed throughout the cortical plate, which contains maturing deep layer neurons, and in migratory upper layer neurons. By E18.5, Csde1 expression is restricted to the upper cortical plate, and is absent in layer VI. In layer V, Csde1 expression is restricted to the pial half. This indicates that Csde1 expression may influence the transition between deep layer and upper layer neuron fate. In primary NPC cultures, knockdown of Csde1 shifts neuronal identity towards layer V Ctip2+ fates. The expression of Csde1 in layer V and the upper layers of the cortex combined with its influence on Ctip2+ and Satb2+ subtypes indicates that Csde1 may fine-tune the expression of these subtypes

within the cortex. This could occur by either repression of Satb2+ and Tbr1+ or through promotion of Ctip2+ expression (Figure 26).

Little is known about how the transition between deep and upper layer neuronal fates is controlled, and primarily this work focuses on neural progenitor fate switching (Toma and Hanashima, 2015). Information on how neuronal subtype specification is altered post-mitotically remains sparce, although post-mitotic neurons continue to refine their molecular identity for several days following their birth (Anda et al., 2016; Oishi et al., 2016). These post-mitotic neurons can be reprogrammed using ectopic transcription factor expression, and remain susceptible to re-specification in the early post-natal window (De la Rossa et al., 2013a). This indicates that disruptions in the balance of transcription factors in the developing cortex could prolong or alter neuronal subtype assignment in the cortex. This could delay or prevent final subtype assignment in the cortex, and could result in increased overlap of subtype specific characteristics. For example, post-mitotic induction of Fezf2 expression in layer IV neurons results in acquisition of layer V projection characteristics, without an increase in layer V molecular marker Ctip2 (De la Rossa et al., 2013b). This altered subtype assignment is seen in other mouse models of ASD. Copy number variations of chromosomal region 16p11.2 are associated with ASD (Fernandez et al., 2010; Pucilowska et al., 2015) and mouse models of the 16p11.2 deletion show altered cortical neuron subtype distribution, similar to our observations of $Csde1^{KO/+}$ (Pucilowska et al., 2015).

In vivo, the effect of Csde1 on neuronal subtype also shows alteration of Ctip2+ neurons. At E18.5, Ctip2+ neurons are found spread into the upper layers of the CP in $Csde1^{KO/+}$ embryos. Conversely, Satb2+ neurons are spread into layer V, resulting in increased co-expression of Ctip2 and Satb2 (Figure 24). Layer VI Tbr1+ neurons are extended towards the pial surface, also

resulting in increased co-expression of Ctip2 and Tbr1. This indicates that some aspect of the layer V transition may be disrupted in Csde1$^{KO/+}$ embryos. This difference in distribution is subtle and methods for counting and assessing distribution are highly susceptible to differences in cortical region and immunostaining efficiency. As a result, further analysis of these perturbations of subtype distribution must be assessed for consequences on neuronal function and connectivity.

This layer V transition is disrupted in the post-natal Csde1$^{KO/+}$ environment as well. At P7, Ctip2+ cells are more abundant, and are expressed into Layer VI (Figure 22). Co-expression of Ctip2 is increased in the Layer VI Tbr1+ population and the upper layer Satb2+ population (Figure 25). This increased co-expression is not reciprocal, indicating that Ctip2 expression may be increased at the expense of the normal Tbr1+ and Satb2+ neuron population. Although this co-expression is abnormal, how this affects overall neuron function is not addressed in this study. To determine if this co-expression and incomplete molecular subtypes affect neuron function, assessment of cell morphology, axon projections and synaptic function is required.

The heterozygous loss of Csde1 in the cortex shows incomplete subtype assignment, delaying final maturation of cortical neurons. This is consistent with previous studies of ASD, which implicate circuit formation and neuronal connectivity as the major regulators of ASD, rather than overall cortical morphology (Zikopoulos and Barbas, 2013). This circuit disruption is subtle overall in the cortex but could have long reaching consequences for overall connectivity in the brain. For example, incomplete Ctip2 expression in Fezf2 deficient cortices, prevents formation of subcerebral connections to the spinal cord (Chen et al., 2008). Co-expression of neuronal transcription factors may therefore prevent canonical axon projections. It has been shown that delays in migration and subtype assignment prevent axonal projections from reaching

their canonical targets (Martineau et al., 2018; Martynoga et al., 2012; Molyneaux et al., 2007). Axonal projection and migration of cortical neurons is temporally regulated by environmental signalling, and delay of assignment may cause these growths to miss their critical projection period. Additionally, previous work shows that Csde1 deficient neurons have reduced axon growth, and reduced synaptic function through disruption of the Wnt/β-catenin pathway, however how this Csde1 mediated Wnt signalling affects neurons *in vivo* is unknown (Guo et al., 2019). Together, altered subtype identity, combined with reduced synaptic potential could disrupt circuit formation in the brain.

The mechanism of Csde1 in the specification of neurons is unknown. Previous work shows that Csde1 interacts with other ASD related RNAs, including mRNA of RBPs FMRP (Fragile-X Mental Retardation Protein) and RBFOX (RNA Binding Fox-1 Homolog) (Guo et al., 2019). This interaction with secondary RBPs indicates that RNA regulation may be crucial for normal cortical development. For example. abnormal RNA editing through FMRP is dysregulated in ASD patient samples (Tran et al., 2019). Although Csde1 is associated with additional ASD related target RNAs in the postnatal (P15) environment, the variability and alteration of Csde1 targets by secondary proteins means that Csde1 may bind different RNA targets in the developing cortex (Guo et al., 2019). As a result, additional work on the role of Csde1 binding must be done in the developmental cortex to determine the mechanism of Csde1 in neuronal subtype specification.

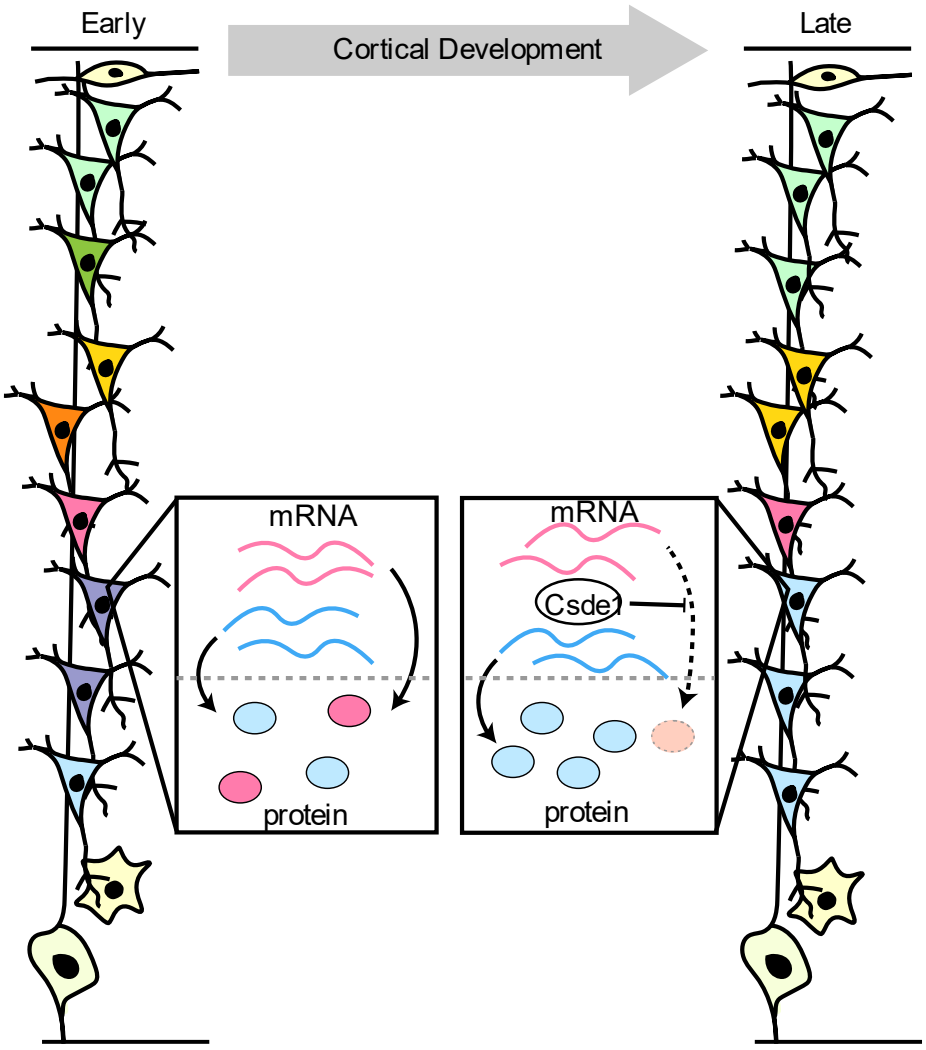

Figure 26. Csde1 fine tunes neuronal subtype in the cerebral cortex. Mixed identity neurons (represented as purple, orange and green) are present in the early cortex. These mixed identity neurons express mRNA and protein for multiple subtype specific transcription factors (represented in pink and blue). As neurons mature, mRNA translation is tailored to restrict neuron identity (represented as blue, pink, and yellow neurons). Csde1 alters the post-transcriptional landscape by either repression (blunt arrow) or enhancement of mRNA translation, allowing final subtype assignment.

ASD is a spectrum disorder, where strengths and deficits of autistic individuals varies by case. This may be a result of the subtle and variable nature of change in the cortical landscapes. This is indeed what I see in Csde1$^{KO/+}$ mice, where changes in subtype specification may affect variable change on the overall connectivity of the brain depending on the location and timing of disruption. This supports ASD as a genetic disorder with variable causative factors and overall presentation (de la Torre-Ubieta et al., 2016).

Chapter 8: Future Directions

This project shows that Csde1 affects neuronal subtype assignment in the murine cortex. Continuing research into the role of Csde1 will focus on the functional consequences of disrupted subtype assignment, as well as potential mechanisms.

8.1 Neuronal connectivity of Csde1 deficient neurons

Alterations in distribution and incomplete subtype assignment may affect neuronal connectivity. Loss of subtype identity is known to disrupt axonal projection (Alcamo et al., 2008; Chen et al., 2008; Hevner et al., 2001). It is therefore likely that disruption of subtype in the $Csde1^{KO/+}$ cortex alters or delays cortical neuron projection. The mixed subtype identities of cortical neurons may exhibit abnormal projection profiles, or prevent integration into major neuronal circuits. In addition, Csde1 is known to affect synaptic function in primary neurons *in vitro* (Guo et al., 2019). To examine these possibilities, labelling of axon projections using lipophilic or viral-retrograde tracing can be assessed to determine if cortical neurons reach their intended target.

Importantly, Csde1 is known to alter migration of interneurons in the cerebellum, and thus may alter interneuron integration in the cortex as well (Kobayashi et al., 2013). Alterations in excitatory/inhibitory balance are known to contribute to epilepsy, which shows high co-morbidity with ASD (Bozzi et al., 2018). As a result, the effect of Csde1 on interneuron maturation and migration cannot be discounted and must be examined in the cortex. Using interneuron specific markers, the presence and distribution of interneurons in the cortex can be examined.

8.2 Behavioural deficits in Csde1$^{KO/+}$ model

To determine if the changes in the neuronal subtypes and overall cortical landscape cause functional deficits, the behaviour of Csde1$^{KO/+}$ mice will be examined.

I expect that Csde1$^{KO/+}$ mice will exhibit ASD associated behaviours. This includes repetitive behaviour, insistence of sameness and social deficits. For example, in mouse models of ASD, self-grooming behaviour is increased in duration and frequency. Additionally, ASD models resist change and take longer to unlearn patterned behaviours such as maze solving and novel object exploration. These ASD model mice also preferentially spend time with familiar mice over novel mice compared to their control counterparts (Silverman et al., 2010). These are reflective, but not comprehensive tests to assess ASD-like behaviours in mice, and it is important to recall that ASD is a spectrum disorder with variable presentation.

To assess the overall effect of heterozygous Csde1 knockout beyond the changes in the cortical neuron landscape, I will examine the changes in behaviour of these mice compared to their wildtype littermates.

8.3 Molecular targets of Csde1 binding

In order to determine the mechanism of Csde1 in the cortex, I must determine the RNA targets of Csde1. To this end, using RNA-immunoprecipitation and next generation sequencing, I can compile a library of RNA binding targets. Previous studies have determined binding targets of Csde1 in other tissue types. However, given the diverse and variable RNAs found within the developing cortex, defining specific targets in the embryonic cortex is required.

Although knowing the RNA binding targets is essential to understanding how Csde1 influences neuronal subtype, it cannot tell us how Csde1 affects the translation of these transcripts. To do this, I can use polysome profiling of the cortex (Kedia et al., 2021). Using $Csde1^{KO/+}$ mice I can compare the translational landscapes and determine how loss of Csde1 changes translational efficiency of key RNA targets.

Together, these can provide insight into the mechanism of Csde1 and its relation to subtype assignment.

Chapter 9: Conclusion

Cortical development is a complex process in which regulation of NPCs and neurons must be balanced to generate hundreds of distinct cell types. Transcriptional and post-transcription regulation within this vast cortical landscape provide a complex regulatory network which governs development. Here, I show that during corticogenesis, RNA binding protein Csde1 regulates NPC differentiation and neuronal subtype specification.

This project shows that Csde1 expression is carefully regulated at the transcriptional and post-transcriptional levels in the developing cortex, and that these levels are important for Csde1 function. Loss of Csde1 results in abnormal subtype assignment within the cortex, altering neuronal distribution and increasing incomplete neuronal differentiation. Disruptions in Csde1 are associated with ASD, indicating that this translational mechanism has functional consequences on overall brain development.

This study provides a phenotypic view of how disruption of Csde1 contributes to neurodevelopmental disorders, such as ASD.

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