Mechanisms of Post-Transcriptional Control in Mammalian Neural Stem Cells

by

Siraj Zahr

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Institute of Medical Science, University of Toronto

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Doctor of Philosophy
Institute of Medical Science
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2019

Abstract

The diverse types of neurons that populate the mammalian forebrain are the fundamental requirement for the assembly of complex circuitry. However, the mechanisms regulating the genesis of these distinct neuronal populations from neural precursor cells (NPCs) are still not well understood. In this thesis, I address the question of whether post-transcriptional mechanisms regulate neuronal differentiation and fate specification within the developing forebrain. First, I show that embryonic NPCs are transcriptionally primed to make diverse neuronal subtypes, and that a Pum2-4E-T complex represses the translation of neuronal specifier mRNAs to ensure the appropriate temporal specification of daughter neurons. Second, I show that 4E-T mediated translational repression continues to act as a critical regulator of NPC maintenance and differentiation at postnatal stages. Based on the work presented in this thesis, I propose that translational repression mechanisms act to ensure the appropriate differentiation of transcriptionally primed NPCs in response to rapidly changing environmental cues.
Acknowledgements

During my PhD, I've grown in more ways than I can clearly pinpoint. Admittedly, the process wasn't always free of discomfort and challenges, but overall was invaluable and rewarding.

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I am forever indebted to my family. Mom and Dad, I increasingly realize how lucky I am to have parents that are not only supportive, but who are open-minded and value intellectual pursuits. Omar and Nina, as far as older siblings are concerned, you aren't that bad I guess.

Tina, I can't really contemplate what graduate school would have been like without you. Thank you for everything.

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Publications and Author Contributions

Chapter 4 is a modified version of the published manuscript "A Translational Repression Complex in Developing Mammalian Neural Stem Cells that Regulates Neuronal Specification" in Neuron 2018, 97: 520-537. Contributions to this published manuscript are as follows: Siraj K. Zahr conceptualized, designed, performed, and analyzed most of the experiments and co-wrote the paper. Guang Yang conceptualized, performed, and analyzed the RIP/microarray experiments and contributed to the qRT-PCR, FISH, culture, and co-immunoprecipitation experiments. Hilal Kazan analyzed and validated the RIP experiments. Scott A. Yuzwa and Michael J. Borrett performed and analyzed the scRNAseq experiments and Michael J Borrett participated in FISH analysis. Anastassia Voronova performed and analyzed the qPCRs. David R. Kaplan conceptualized experiments and co-wrote the paper. Freda D. Miller conceptualized and designed experiments, analyzed data, and co-wrote the paper. This work was funded by CIHR grants and the CFREF "Medicine by Design" to Freda Miller and David Kaplan and by an EU FP7 Marie Curie grant to Hilal Kazan. Freda Miller is a Canada Research Chair and was an HHMI Senior International Research Scholar during the course of this work. Siraj K. Zahr was funded by a CIHR MD/PhD studentship, Guang Yang by a Brain Canada Mental Health Fellowship and an HSC Restracomp/CBMH fellowship, Scott A. Yuzwa by OIRM and Lap-Chee Tsui HSC Restracomp postdoctoral fellowships, and Michael J. Borrett by an NSERC Masters studentship. We thank Sarah Burns and Dennis Aquino for technical assistance, Stefano Stifani for the Tle4 antibody, John Vessey for the Pum2 shRNA, and Jing Wang for the CBP phosphomimic expression plasmid.
Chapter 5 is a modified version of an unpublished manuscript. Contributions to this chapter are as follows: Siraj K. Zahr conceptualized, designed, performed, and analyzed most of the experiments. Guang Yang helped generate the mice carrying a floxed allele of the 4E-T locus. Michael J. Borrett performed the scRNAseq experiments. David R. Kaplan conceptualized experiments and co-wrote the manuscript. Freda D. Miller conceptualized and designed experiments, analyzed data, and co-wrote the manuscript.
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<th>Definition</th>
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<tr>
<td>3’ UTR</td>
<td>3’ untranslated region</td>
</tr>
<tr>
<td>4E-T</td>
<td>4E-transporter</td>
</tr>
<tr>
<td>APKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>bHLH</td>
<td>Basic helix-loop-helix</td>
</tr>
<tr>
<td>BDNF</td>
<td>Brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>Bhle22</td>
<td>Basic helix-loop-helix family member e22</td>
</tr>
<tr>
<td>BLBP</td>
<td>Brain-lipid binding protein</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>BP</td>
<td>Basal progenitor, also known as intermediate progenitor (IP)</td>
</tr>
<tr>
<td>BRAT</td>
<td>Brain tumour protein</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2’-deoxyuridine</td>
</tr>
<tr>
<td>Brn1</td>
<td>Brain-Specific Homeobox/POU Domain Protein 1, also known as POU Class 3 Homeobox 3 (Pou3f3)</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CCR4-NOT</td>
<td>Carbon catabolite repression 4 (CCR4)–negative on TATA-less (NOT)</td>
</tr>
<tr>
<td>CC3</td>
<td>Cleaved caspase 3</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>CDS</td>
<td>Coding sequence</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>CLIP</td>
<td>Cross-linking immunoprecipitation</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CP</td>
<td>Cortical plate</td>
</tr>
<tr>
<td>CPEB</td>
<td>Cytoplasmic polyadenylation element binding protein</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>Ctip2</td>
<td>COUP-TF-interacting protein 2, also known as B Cell CLL/Lymphoma 11B (Bcl11b)</td>
</tr>
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<td>Cux1</td>
<td>Cut-Like Homeobox 1</td>
</tr>
<tr>
<td>Cux2</td>
<td>Cut-Like Homeobox 2</td>
</tr>
<tr>
<td>DAVID</td>
<td>Database for Annotation, Visualization and Integrated Discovery</td>
</tr>
<tr>
<td>Dazl</td>
<td>Deleted in azoospermia-like</td>
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<tr>
<td>Dcp1</td>
<td>Decapping Enzyme 1</td>
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<tr>
<td>Dcx</td>
<td>Doublecortin</td>
</tr>
<tr>
<td>DDX6</td>
<td>DEAD box protein 6</td>
</tr>
<tr>
<td>DG</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>Diap3</td>
<td>Diaphanous homolog 3</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>D. melanogaster</td>
<td>Drosophila melanogaster</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day. E.g.: E13, embryonic day 13.</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EIF4E</td>
<td>Eukaryotic initiation factor 4E</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EIF4G</td>
<td>Eukaryotic initiation factor 4G</td>
</tr>
<tr>
<td>Emx2</td>
<td>Empty spiracles homebox 2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-regulated Kinase</td>
</tr>
<tr>
<td>FC</td>
<td>fold change</td>
</tr>
<tr>
<td>Fezf2</td>
<td>FEZ family zinc finger 2</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FMRP</td>
<td>Fragile X mental retardation protein</td>
</tr>
<tr>
<td>Foxp2</td>
<td>Forkhead box protein 2</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-aminobutyric acid</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>GLAST</td>
<td>Glutamate aspartate transporter</td>
</tr>
<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>G1</td>
<td>Gap1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap2</td>
</tr>
<tr>
<td>Hes5</td>
<td>Hairy and enhancer of split 5</td>
</tr>
<tr>
<td>HuR</td>
<td>Human antigen R also known Elavl1, embryonic lethal abnormal vision-like 1</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>INM</td>
<td>Interkinetic nuclear migration</td>
</tr>
<tr>
<td>IP</td>
<td>Intermediate progenitor also known as basal progenitor (BP)</td>
</tr>
<tr>
<td>IZ</td>
<td>Intermediate zone</td>
</tr>
<tr>
<td>JNK</td>
<td>C-jun N-terminal kinase</td>
</tr>
<tr>
<td>LGE</td>
<td>Lateral eminence</td>
</tr>
<tr>
<td>Lhx2</td>
<td>LIM/homeobox protein 2</td>
</tr>
<tr>
<td>Lix1</td>
<td>Limb and CNS Expressed 1</td>
</tr>
<tr>
<td>Lmo4</td>
<td>LIM domain only 4</td>
</tr>
<tr>
<td>LV</td>
<td>Lateral ventricle</td>
</tr>
<tr>
<td>M7G</td>
<td>7-methylguanosine cap</td>
</tr>
<tr>
<td>Mash1</td>
<td>Mammalian achaete scute homolog-1 also known as Ascl1</td>
</tr>
<tr>
<td>MGE</td>
<td>Medial ganglionic eminence</td>
</tr>
<tr>
<td>miRNA</td>
<td>MicroRNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>mTor</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MZT</td>
<td>Maternal to zygotic transition</td>
</tr>
<tr>
<td>NESC</td>
<td>Neuroepithelial stem cells</td>
</tr>
<tr>
<td>NeuroD</td>
<td>Neurogenic differentiation factor 1</td>
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<tr>
<td>Neurog1</td>
<td>Neurogenin 1</td>
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<tr>
<td>Neurog2</td>
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<tr>
<td>Nkx2.1</td>
<td>Thyroid transcription factor 1</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural precursor cell</td>
</tr>
<tr>
<td>NPY-R</td>
<td>Neuropeptide Y receptor</td>
</tr>
<tr>
<td>NSC</td>
<td>Neural stem cell</td>
</tr>
<tr>
<td>OB</td>
<td>Olfactory bulb</td>
</tr>
</tbody>
</table>
**V-SVZ**: ventricular-subventricular zone  
**Wnt**: Wingless-related integration site
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Chapter 1. Introduction

1.1 Overview of neurogenesis in the embryonic murine cortex

The mammalian neocortex is the most evolutionarily recent region of the central nervous system (CNS), and is responsible for processing sensory information, controlling motor output, and mediating higher-order cognitive functions. The human neocortex consists of four lobes and is patterned by sulci (furrows/depressions) and gyri (ridges). While mice have analogous functional organization, they do not have sulci and gyri and are thus termed lissencephalic or smooth-brained. In mice, neocortical projection neurons are generated from neural precursor cells (NPCs) between embryonic day (E)10.5 and E17, followed by astrocytes and oligodendrocytes (Gauthier-Fisher and Miller, 2013). Defects in the number and/or subtypes of neurons generated during this critical developmental window can lead to inappropriate neural circuit assembly and contribute to neurodevelopmental disorders. In fact, these alterations have been linked to several disorders including Autism, Down Syndrome, epilepsy, and schizophrenia (Stoner et al., 2014; Mao et al., 2009; DiCocco-Bloom et al., 2006; Becker et al., 1991; Becker et al., 2006). Therefore, elucidating the mechanisms underlying appropriate neurogenesis and neuronal specification can provide insight into how such disorders may arise, and the knowledge gained can lead to novel therapeutic strategies to enhance neural repair (Wang et al., 2012; Dadwal et al., 2015).

Our lab uses the embryonic NPCs that generate neurons in the murine cerebral cortex as a model system to study brain development. This is the model of choice for three major reasons. First, the timing of neuron, astrocyte, and oligodendrocyte genesis has been well
characterized by several groups (Kriegstein and Alvarez-Buylla, 2009; Taverna et al., 2014). Second, the temporal order of various cell types generated from NPCs in culture largely recapitulate in vivo development (Qian et al., 2000; Shen et al., 2006). Third, the cortex is superficially located in the embryo and thus experimentally amenable to electroporation and dissection.

**Formation of the dorsal telencephalon**

The neocortex ultimately develops from a region known as the dorsal telencephalon. The formation of the telencephalon initiates after signals originating from underlying mesodermal tissue signals the ectoderm above it to proliferate and acquire neural potential, forming a thickened structure known as the neural plate (Sadler, 2012). This neural plate begins to invaginate dorsally and connect ventrally, pinching off the ectoderm to form the neural tube (Sadler, 2012; Wilde et al., 2014). The lumen of the neural tube is known as the neural canal, which develops into the four ventricles of the brain and central canal of the spinal cord. The two ventricles that will be repeatedly mentioned throughout this thesis are the lateral ventricles (LV). The telencephalon forms from the rostral most pole of the neural tube. Around E8-9 in mice, the neuroectodermal or neuroepithelial stem cells (NESCs) that constitute the telencephalon are mitotically-active and display various epithelial properties. At this stage, gradients of diffusible morphogens such as bone morphogenetic proteins (BMPs), Wingless (WNTs), fibroblast growth factor 8 (Fgf8) and Sonic Hedgehog (Shh) pattern and organize the neural tube and telencephalon along both a dorso-ventral and antero-posterior axis (Grove et al., 1998; Fukuchi-Shimogori and Grove, 2001; Greig et al., 2013). These signaling molecules induce the expression of transcription factors in a region-specific manner. Transcription factors required to confer a dorsal telencephalic identity include Pax6,
Emx2, Lhx2 and Gli3, while Dlx1/2/5, Gsh2, Mash1, Lhx6, and Nkx2.1 confer a ventral telencephalic identity (Walther and Gruss, 1991; Gulisano et al., 1996; Grove et al., 1998; Sussel et al., 1999; Lee and Jessel., 1999; Toresson and Campbell, 2001; Yun et al., 2003; Corbin et al., 2003; Chou et al., 2009). In fact, Emx2/Pax6 double mutants do not form cortical structures and the entire telencephalon is ventralized (Muzio et al., 2002; Bishop et al., 2002). The ventral telencephalon can be further subdivided into the medial (MGE), caudal (CGE), and lateral ganglionic eminences (LGE). During embryogenesis, glutamatergic excitatory neurons are generated from dorsal neural precursors, while GABAergic inhibitory interneurons are generated from ventral neural precursors residing in the GE (Molyneaux et al., 2007). Throughout this thesis, I will focus on the dorsal telencephalon and dorsal neural precursor cells as they are most relevant to my thesis. I will use the term “neural precursor cells (NPCs)” to broadly describe the mixture of mitotic cells that generate the cortex.

1.1.1 Neural precursor cells within the developing cerebral cortex

Neuroepithelial stem cells (NESC)

The telencephalon begins as a pseudostratified neuroepithelial cell layer consisting of mitotically-active neural progenitor cells known as NESC. This cortical layer, known as the ventricular zone (VZ), forms around E8-9. NESC are polarized along an apico-basal axis and display classical epithelial characteristics (Huttner and Brand, 1997). For instance, Prominin-1, tight-junctions, and adherens junctions are found in the apical portion of the cell (Weigmann et al., 1997; Corbeil et al., 2001; Aaku-Saraste et al., 1996; Zhadanov et al., 1999; Manabe et al., 2002), while integrin α6 is associated with the basal end (Wodarz and
Adherens junctions are required for maintenance of apico-basal polarity (Zhadanov et al., 1999). NESCs have a bipolar morphology, extending processes towards and making contact with both the apical (ventricular) and basal (pial) aspects of the neural tube. Importantly, these NESCs divide symmetrically to expand the precursor pool (Chenn and McConnell, 1995; McConnel, 1995; Rakic, 1995).

**Radial Glial Cells (RGCs)/ Radial Precursors (RPs)**

NESCs undergo a transition to radial glial cells (RGCs) or radial precursors (RPs) between E10-12 prior to the onset of cortical neurogenesis (Hartfuss et al., 2001; Gotz et al., 2002; Noctor et al., 2002; Malatesta et al., 2003; Anthony et al., 2004). They were originally named radial glia due to their expression of classical astrocytic markers such as glial fibrillary acidic protein (GFAP) (in humans but not mice) and their long radial fibres that serve as scaffolds to guide the migration of newborn neurons in the cortex (Levitt and Rakic, 1980; Rakic 1972; Sancho-Tello et al., 1995). Subsequent time-lapse imaging studies of retrovirally labelled RPs showed that they divide and give rise to neurons that migrate along their radial fibres. Therefore, RPs themselves are the precursors to neurons and glia in the embryonic cortex in addition to serving as a migratory scaffold (Noctor et al., 2001). Subsequent studies showed that RPs are multipotent at a population level, sequentially giving rise to neurons followed by glia (Noctor et al., 2002). I will refer to RGCs/RPs as RPs throughout the thesis, consistent with their role as neural precursors during embryogenesis.

Like their NESC predecessors, RPs are polarized epithelial cells possessing long apical and basal processes. This basal process becomes greatly elongated as the cortex grows in size (Rakic, 2003). Several notable changes occur during the transition of NESCs to RPs.
RPs gain astrocytic features such as the expression of glial proteins glutamate aspartate transporter (GLAST), brain lipid-binding protein (BLBP), radial glial cell marker-2 (RC2), and vimentin (Shnitzer et al., 1981; Malatesta et al., 2000; Hartfuss et al., 2001) and the presence of glycogen granules (Choi, 1981). Tight junction components are downregulated during RP generation, while adherens junctions ZO-1 and N-cadherin are upregulated (Aaku-Saraste et al., 1996). Importantly, RPs express the important transcription factor Paired box protein 6 (Pax6) that is involved in progenitor cell cycle progression, proliferation, and neurogenesis (Quinn et al., 2007; Manuel et al., 2015). Extrinsic factors that regulate the transition to RPs include Notch1 and Fgf10 (Gaiano et al., 2000; Sahara and O’Leary, 2009). Unlike NESCs, which are limited to symmetric divisions, RPs can divide symmetrically or asymmetrically to give rise to neurons directly or indirectly via an intermediate/basal progenitor (IP/BP) (Figure 1).

Figure 1. Direct versus Indirect Neurogenesis.
(A,B) Radial precursors (RPs) can give rise to a post-mitotic neuron directly (A) or indirectly via an intermediate progenitor (IP) cell (B). IPs provide a means to amplify the neuronal output per neurogenic division.

**Intermediate/basal progenitors (IPs/BPs)**

RPs can divide asymmetrically to generate a copy of themselves and a post-mitotic neuron (direct neurogenesis). Alternatively, they can divide asymmetrically to generate a copy of themselves and an IP, which in turn can divide once or twice to produce post-mitotic neurons (indirect neurogenesis) (Figure 1). It is widely thought that IPs function as an evolutionary means of expanding the cortex by enabling the generation of a larger amount of neurons (Martinez-Cerdeno et al., 2006; Arnold et al., 2008; Sessa et al., 2008). IPs begin to be generated around E11 at the onset of neurogenesis, and populate the space immediately basal to the VZ known as the subventricular zone (SVZ) (Arnold et al., 2008; Sessa et al., 2008). As they form, they move basally into the SVZ and retract their apical and basal processes. Unlike RPs, IPs are not anchored to the apical or basal surfaces, are restricted to making neurons, lack polarity, and divide exclusively in a symmetric fashion (Haubensak et al., 2004; Noctor et al., 2004). The majority of these divisions yield two daughter neurons, but symmetric proliferative divisions can also occur (Noctor et al., 2008; Attardo et al., 2008, Haubensak et al., 2004). They have limited self-renewal capacity, undergoing at most two rounds of symmetric divisions (Englund et al., 2005). Importantly, IPs express the transcription factor T-Box brain protein 2 (Tbr2) which is critical for IP formation from RPs
(Englund et al., 2005; Arnold et al., 2008; Sessa et al., 2008). They also express other markers such as the non-coding RNA Svet1 (Tarabykin et al., 2001), the transcription factors Cux1 and Cux2 (Cubelos et al., 2008; Nieto et al., 2004; Zimmer et al., 2004) and Neurogenin2 (Miyata et al., 2004). IPs contribute to neurons residing in all cortical layers, generating between 70-80% of all glutamatergic excitatory neurons in the murine cortex, and do not persist past the embryonic neurogenic period (Kowalczyk et al., 2009; Vasistha et al., 2015). Moreover, loss of IPs results in a reduction of neurons in all layers (Sessa et al., 2008).

1.1.2 Cortical Projection Neurons

The neocortex consists of two major neuronal cell types: projection neurons and interneurons. Projection neurons account for 70-80% of neurons within the cortex (Lodato and Arlotta, 2015). Due to their triangular shaped cell bodies (somas), they are often referred to as pyramidal neurons. They primarily secrete the excitatory neurotransmitter glutamate, thus often referred to as excitatory projection neurons. They are capable of extending axons over very large distances up to the various levels of the spinal cord. As previously mentioned, projection neurons are generated by precursors in the dorsal (pallial) telencephalon.

Cortical interneurons comprise 15-30% of neurons in the neocortex, make local connections, and use the inhibitory neurotransmitter GABA. In contrast to projection neurons, they are generated from precursors in the ventral (subpallial) telencephalon (from the GE), after which they migrate tangentially into the developing cortex (Lodato and Arlotta, 2015). They are further classified into various interneuron subtypes. I will not be
discussing cortical interneuron biology and diversity further, as they are not relevant to my thesis (for a review on the topic, see Lim et al., 2018). Instead, I will focus on the properties of projection neurons generated embryonically.

**Projection neuron arrangement and diversity within the neocortex**

The projection neurons that comprise the mature cortex are arranged into six layers (I-VI) and differ with regard to their morphology, connectivity, and gene expression, and are essential for circuitry assembly. The first neurons generated from RPs migrate away from the VZ to generate the preplate. Later-born neurons migrate into the preplate, dividing it into the marginal zone (MZ) and subplate (SP) and forming the cortical plate (CP) between them. Subsequently, projection neurons are generated in an inside-out fashion to populate the six-layers of the cortex; earlier born neurons populate the deepest of the six cortical layers (V-VI), while later born neurons populate progressively more superficial layers (II-IV) (Figure 2) (Rakic 1974; Mariani et al., 2012; Espuny-Camacho et al., 2013; Greig et al., 2013).
Figure 2. Cortical neurogenesis occurs in an inside-out fashion

(A-C) Cortical projection neurons are generated in an inside-out fashion to populate the six-layers of the cortex. Earlier born neurons (shown in purple) populate the deepest of the six cortical layers (V-VI), while later born neurons (shown in red) populate progressively more superficial layers (II-IV). These layers contain distinct neuronal subtypes that differ based on morphology, electrophysiological activity, axonal connectivity, and gene expression.
Projection neurons have historically been classified based on laminar location of their somas, dendritic morphology, electrophysiological activity, and axonal connectivity (Molyneaux et al., 2007; Migliore and Shepherd, 2005). To simplify matters, they can be broadly classified into two categories: intracortical projection neurons (commissural and associative projection neurons) and corticofugal projection neurons (corticothalamic and subcerebral projection neurons) (Molyneaux et al., 2007; Lodato et al., 2014). Intracortical neurons are predominantly located in upper cortical layers (layer II-III) and can project their axons within one cortical hemisphere (associative projection neurons) or across the midline towards the contralateral hemisphere (commissural projection neurons). The majority of commissural projection neurons project their axons via the corpus callosum (callosal projection neurons) while a smaller proportion travel through the anterior commissure. These neurons play a role in bilateral integration of information (Greig et al., 2013; Lodato et al., 2014). Corticofugal projection neurons on the other hand are primarily located in deep cortical layers (layer V-VI; infragranular layers) and project axons to distal subcortical targets such as nuclei in the thalamus, brain stem, and spinal cord. They are further subdivided into corticothalamic neurons which reside in layer VI and project towards thalamic nuclei, or subcerebral projection neurons which reside in layer V and project axons towards nuclei in the midbrain (corticotectal neurons), brain stem (corticopontine neurons), and spinal cord (corticospinal motor neurons) (Molyneaux et al., 2007; Lodato et al., 2014).

In addition to laminar location and axonal connectivity, gene expression is another useful framework to classify projection neurons. Previous studies have transcriptionally profiled purified populations of projection neurons in various cortical layers and have identified important markers (Arlotta et al., 2005; Doyle et al., 2008; Heiman et al., 2008;
For instance, Fezf2, Diap3, Ctip2/Bcll1b, Sox5, Tle4, and Ldb2 are classical deep layer neuron-specific genes that are excluded from superficial layer callosal projection neurons (Arlotta et al., 2005; Lodato et al., 2014). On the other hand, Tle1, Tle3, Lhx2, Cux1, Cux2, Cited2, Brn1/2, PlexinD1, and Satb2 are important genes expressed predominantly in superficial layer callosal projection neurons but relatively absent from deep layer subcerebral projection neurons (Molyneaux et al., 2009). A recent study performed RNA-sequencing on microdissected cortical layers from the mouse primary somatosensory cortex and identified about 5,835 differentially expressed protein-coding genes across the different layers. Interestingly, they also observed 66 lincRNAs that were differentially expressed, as well as 1646 genes with differential alternative splicing (Belgard et al., 2011). These findings suggest that mechanisms beyond transcriptional control are likely important to generate the full diversity of neuronal subtypes. As I will discuss later, some of the subtype/layer specific markers have been shown to play key roles in the specification of different neuronal subtypes.

It is important to note that these population-based studies do not fully account for the cellular and molecular heterogeneity that is beginning to be elucidated with single-cell profiling approaches. Interestingly, recent single-cell RNA sequencing studies of the cortex have identified “intermediate” clusters in addition to sharply defined clusters that map to known projection neuron subtypes (Zeisel et al., 2015; Tasic et al., 2016; Lake et al., 2016; Johnson and Walsh, 2017) It remains to be seen whether these clusters represent novel projection subtypes, different states of the same subtype, or experimental/computational
artifacts. A more provocative interpretation is that these clusters may represent plasticity of adult neuronal identity (Johnson and Walsh, 2017).

1.1.3 Models of Neurogenesis

Classical \(^3\)H-thymidine labelling studies were the first to show that NPCs give rise to deep layer neurons before superficial layer neurons (Angevine et al., 1961; Rakic, 1974). More recent \textit{in vivo} lineage tracing studies have since shown that at both a population and clonal level, early RPs are multipotent and sequentially give rise to deep and superficial layer neurons, followed by glia (Luskin et al., 1988; Guo et al., 2013; Gao et al., 2014; Eckler et al., 2015). How are these different layer pyramidal neurons generated from a pool of NPCs during embryonic neurogenesis? Until recently, the prevailing model to explain this stereotypical production of neurons was that an initial pool of multipotent NPCs undergoes progressive restriction of progenitor fate throughout neurogenesis. Classical heterochronic transplantation experiments performed in ferrets showed that early progenitors transplanted into an older cortex in which superficial neurons are being made are capable of generating neurons of all layers (i.e. still capable of giving rise to early deep-layer neurons). Conversely, late progenitors (destined to give rise to layer II/III neurons) fail to give rise to earlier born-deep layer neurons when transplanted into a younger cortical niche (Frantz and McConnell, 1996; Desai and McConnell, 2000).

Recent work has challenged this long-standing dogma that RPs become fate restricted as they progress through successive temporal states to generate deep and superficial layer neurons (Oberst et al., 2018). Using a FlashTag (FT) method to pulse-label and specifically isolate E15 RPs, followed by heterochronic transplantation of these tagged RPs into younger
E12 embryos, the authors showed that older RPs can re-enter a previous molecular state to generate earlier-born neurons (Oberst et al., 2018). In contrast, IPs lack this plasticity and lose the ability to produce earlier born neurons when transplanted into a younger niche (Oberst et al., 2018). The difference between these recent findings and earlier transplantation studies stems from the fact that the latter involved the transplantation of a heterogeneous pool of thymidine-labelled progenitors (Frantz and McConnell, 1996; Desai and McConnell, 2000). Given that the majority of progenitors in the late ferret cortex are IPs, the fate plasticity of RPs would be concealed by the predominance of fate-restricted IPs (Frantz and McConnell, 1996; Desai and McConnell, 2000). Thus, although RPs progress through successive temporal states during corticogenesis, they do so with negligible fate restriction (Telley et al., 2018; Oberst et al., 2018). Consistent with this discovery, a recent single-cell study showed that once the influence of cell cycle genes are regressed out, E13 and E15 RPs are strikingly similar at the transcriptional level (Yuzwa et al., 2017).

1.2 Molecular mechanisms of neurogenesis and neuronal subtype specification

The precise balance between RP self-renewal and differentiation in the developing neocortex involves the interaction of intrinsic cellular programs with a multitude of environmental cues. As I have described, the neuronal progeny that arise from the asymmetric divisions of RPs are far from homogeneous. Therefore, additional regulatory processes must occur in conjunction with differentiation in order to correctly specify particular neuronal subtypes. Due to space constraints, I will not cover most of this
regulation (for reviews on the topic, refer to Miller and Gauthier-Fisher, 2013; Taverna et al., 2014; Paridaen and Huttner, 2014). Instead, I will highlight a few intrinsic regulators that play essential roles in cortical development such as Pax6 and the basic helix-loop-helix (bHLH) transcription factors, in order to provide necessary background to my work. I will also describe some of the extrinsic regulators that feed onto intrinsic programs, such as Notch, fibroblast growth factor, and neurotrophin signaling, in order to highlight the importance of the microenvironment in regulating NPC fate decisions. However, the main emphasis will be on regulatory mechanisms that specify particular neuronal subtypes, as this is most relevant to the work presented in Chapter 4.

1.2.1 Intrinsic mechanisms regulating neurogenesis

Transcriptional and epigenetic regulation

As previously mentioned, transcription factors such as Pax6 and Emx2 are induced in response to diffusible morphogens and specify dorsal cortical identity, while repressing the expansion of ventral regions (Muzio et al., 2002). In addition to their roles in regionalization, several transcription factors subsequently act to regulate the balance between NPC self-renewal and differentiation. Beyond its role in regionalization of the dorsal cortex, the transcription factor Pax6 acts as a master regulator of NPC cell cycle progression and neurogenesis. NPCs cultured from Pax6\textsuperscript{-/-} mutant cortices at E12.5 display accelerated proliferation, while NPCs cultured from mutant cortices at E15.5 display slowed proliferation, suggesting that Pax6’s function is not only cell-autonomous but context-dependent (Estivil-Torrus et al., 2002). Loss of Pax6 \textit{in vivo} causes shortening of cell cycle duration, but not in all cortical regions. This is attributable to the fact that Pax6 expression
levels vary between cortical regions during early corticogenesis and become more uniform with increasing age (Mi et al., 2013). Retroviral overexpression of Pax6 impairs NPC proliferation in vitro and increases neuron formation. Consistent with this, Pax6\(^{-/-}\) mutant cortices have markedly less neurons compared with controls (Heins et al, 2002; Hack et al., 2004; Cartier et al., 2006). Taken together, these studies indicate that Pax6 functions to generally repress NPC cell-cycle progression and promote neuron formation, although it appears to act in a spatiotemporally specific manner.

The basic helix-loop-helix (bHLH) family of transcription factors are some of the most well-studied transcription factors in the context of cortical neurogenesis (Imayoshi and Kageyama, 2014). Some bHLH proteins promote self-renewal of NPCs while others promote neuronal differentiation. bHLH proteins Neurog1 and Neurog2 are transcriptional activators that are expressed in dorsal progenitors during the period of corticogenesis and display proneural activity (Schuurmans et al., 2004). Both gain-of-function and loss-of-function studies have demonstrated their roles in inducing generic neuronal differentiation (Bertrand et al., 2002; Schuurmans et al., 2004; Mattar et al., 2008). They do so by activating the transcription of genes in the dorsal glutamatergic pathway while repressing the expression of *Mammalian achaete scute homolog-1 (Ascl1/Mash1)*, which specifies an alternative ventral, GABAergic neuronal identity (Casarosa et al., 1999; Fode et al., 2000; Parras et al., 2002; Kovach et al., 2013).

Other bHLH proteins such as Hes, Hey, and Id repress proneural genes and in doing so promote NPC self-renewal (Ross et al., 2003; Toma et al., 2000; Bai et al., 2007; Kageyama et al., 2008). Hes1 and Hes5 are expressed by VZ NPCs during cortical neurogenesis, and directly bind and repress the expression of proneural genes such as *Ascl1*...
and Neurog2. In the absence of Hes proteins, proneural genes are upregulated and neurogenesis is promoted (Hatakeyama et al., 2004; Imayoshi et al., 2008). Hes proteins act cooperatively to promote NPC maintenance, thus Hes mutant mice affecting multiple Hes factors display more severe phenotypes compared to single Hes mutants. Hes1/5 double knockout mice show upregulation of proneural proteins resulting in precocious differentiation, depletion of NPCs, and overall disorganization of nervous system structures (Ohtsuka et al., 1999; Hatakeyama et al., 2004). Consistent with this, overexpression of Hes proteins keeps NPCs in an undifferentiated state (Ishibashi et al., 1994; Ohtsuka et al., 2001).

The ability of transcription factors to execute their function requires accessible chromatin sites on DNA. Several epigenetic modifiers that influence chromatin accessibility have been shown to play a role in corticogenesis. One notable example is CREB binding protein (CBP), a histone acetyltransferase (HAT) that interacts and cooperates with several transcription factors. CBP’s HAT activity leads to opening of chromatin at transcriptional activation sites allowing transcription of its downstream targets (Bannister and Kouzarides, 1996). Using CBP+/− mice and a siRNA knockdown strategy, it was shown that CBP promotes the differentiation of NPCs into neurons and glia both in vitro and in vivo via acetylation of histones located at the promoters of proneural genes (Wang et al., 2010). Mutations in the CBP gene account for around half of cases of the genetic disorder Rubinstein-Taybi syndrome that is characterized by severe cognitive dysfunction (Roelfsema and Peters, 2007). Interestingly, these cognitive defects are recapitulated in CBP+/− mice (Wang et al., 2010).
1.2.2 Specification of diverse excitatory neuron subtypes

Rather than simply generating a homogeneous population of neurons, RPs must sequentially give rise to diverse neuronal subtypes that populate the different layers of the cortex. How does neuronal subtype specification occur? As previously discussed, many genes have been identified that specifically label particular neuronal layers/subtypes and are excluded from others (Leone et al., 2008; Molyneaux et al., 2007; Greig et al., 2013). However, a relatively small number of these genes have been characterized for a functional role in specifying neuronal subtypes (Figure 3). I will refer to these particular transcription factors as “specifiers” throughout my thesis.

Specification of deep-layer subcortical projection neurons

The zinc-finger transcription factor (Fezf2) is a critical specifier of subcerebral projection neurons that reside in layer V (Figure 3). It is expressed by VZ progenitors during deep layer neurogenesis and by post-mitotic deep-layer neurons. In Fezf2 knockout mice, subcerebral projection neurons are lost, the expression of subcerebral projection neuron markers is absent, and neurons fail to project their axons towards the brainstem and spinal cord (Inoue et al., 2004; Arlotta et al., 2005; Molyneaux et al., 2005). In the absence of Fezf2, T-box brain protein 1 (Tbr1), a specifier of layer VI corticothalamic projection neurons, is ectopically expressed in many layer V cells (Figure 3). These Tbr1-expressing neurons project their axons to the thalamus (Bedogni et al., 2010; McKenna et al., 2011). Tbr1 is expressed at high levels in layer VI corticothalamic projection neurons and in its absence, corticothalamic projection neurons do not form appropriately and aberrantly express
Fezf2 and Ctip2 (Figure 3). As a result, they inappropriately project axons to subcerebral targets instead of the thalamus (Bedogni et al., 2010; McKenna et al., 2011; Han et al., 2011). These observations are consistent with a model in which Tbr1 and Fezf2 cross-repress one another (Figure 3). Coup-tf-interacting protein 2 (Ctip2, or Bcl11b) is another transcription factor specifier of layer V subcerebral projection neurons that is downstream of Fezf2 (Figure 3). In the absence of Ctip2, subcerebral projection neurons are still born and migrate appropriately to layer V, but their axons are misrouted, defasciculated, and fail to reach the spinal cord (Arlotta et al., 2005). Finally, SRY-box containing gene 5 (Sox5) is a specifier of deep layer corticofugal projection neuron subtypes (Figure 3). It is only expressed in deep-layer (V-VI) neurons and controls the appropriate timing of deep layer neurogenesis by repressing Fezf2 until generation of corticothalamic projection neurons is complete (Lai et al., 2008; Kwan et al., 2008).

**Specification of superficial-layer intracortical neurons**

Deep-layer corticofugal projection neuron specifiers exert their function in large part by repressing upper layer callosal projection neuron fates. For instance, Fezf2 overexpression in layer II/III callosal projection neurons *in vivo* can reprogram them to acquire layer V/VI properties and redirect their axons towards subcortical areas (Chen et al., 2005;2008; Rouaux and Arlotta, 2013). In the absence of Fezf2, neurons acquire upper-layer callosal projection neuron properties; they project their axons across the corpus callosum, display callosal projection neuron electrophysiological properties, and express callosal projection neuron-specific genes (Chen et al., 2005;2008). Special AT-rich sequence binding
protein 2 (Satb2) is a specifier of upper layer callosal projection neurons (Figure 3) (Alcamo et al., 2008; Britanova et al., 2008). In the absence of Satb2, neurons project their axons to subcortical targets instead of across the corpus callosum (Alcamo et al., 2008; Srinivasan et al., 2012). Satb2 acts in part by repressing Ctip2 and thus corticofugal projection neuron identity (Figure 3) (Alcamo et al., 2008; Britanova et al., 2008). Taken together, Sox5, Satb2, Fezf2, Tbr1, and Ctip2 comprise a cross-repressive transcriptional circuit that regulates projection neuron specification (Figure 3).

Although this transcriptional circuit model is elegant and has experimental support, the molecular controls underlying neuronal subtype specification are likely much more complicated. Many additional layer/subtype specific genes have been identified and other transcription factors have since been shown to play critical roles in specification (Belgard et al., 2011; Molyneaux et al., 2015). For example, the POU domain transcription factors Brn1 (Pou3f3) and Brn2 (Pou3f2) proteins are expressed in the VZ/SVZ starting around mid-neurogenesis when superficial layer neurons begin to be generated, and their expression is maintained in neurons of layer II-IV (McEvilly et al., 2002; Sugitani et al., 2002). Brn1/2 double knockout cortices lack upper-layer neurons, while Tle4- and Tbr1-expressing deep layer neurons remain intact (McEvilly et al., 2002; Sugitani et al., 2002). Over-expression of either Brn1 or Brn2 using in utero electroporation during deep-layer neurogenesis results in switching to an upper-layer neuronal fate indicated by the upregulation of upper layer markers (Cux1, Satb2), reduced expression of deep layer markers (Ctip2), and rerouting of axonal projections towards the corpus callosum (Dominguez et al., 2013). Moreover, ablation of Brn1/2- transcriptional activation activity resulted in defects in neurogenesis primarily after E13.5 when superficial-layer neurons are being produced (Dominguez et al., 2013).
Figure 3. Transcriptional model of neuronal subtype specification

Cortical layers I-IV contain intracortical neurons predominantly (layer IV shown in red). Cortical layers V-VI contain corticofugal projection neurons predominantly. Corticofugal projection neurons can be further classified into layer V subcerebral neurons (shown in purple) and layer VI corticothalamic neurons (shown in green). Tbr1 specifies layer VI corticothalamic neurons in part by repressing Fezf2, while Fezf2 acts upstream of Ctip2 to specify layer V subcerebral neurons. Sox5 regulates the timing of deep layer neurogenesis by repressing Fezf2 until the production of layer VI corticothalamic neurons is complete. Satb2 specifies upper layer callosal projection neurons in part by repressing deep-layer neuronal specifiers Ctip2 and Tbr1 (Leone et al., 2008).

1.2.3 Extrinsic cues regulating neurogenesis

While intrinsic factors clearly influence NPC biology, several studies have also highlighted the role of the environment in regulating precursor maintenance, differentiation,
and cell fate decisions. Perturbations of these environmental cues that may occur in the context of neural injury, maternal infections, or genetic conditions, can have a significant impact on RP behavior and neurogenesis (Gauthier-Fisher and Miller, 2013). The elongated radial morphology of RPs provides them with unique access to cues originating from several sources including the meninges, vasculature, newborn neurons, and cerebrospinal fluid (CSF). I will review some of these cues to highlight basic principles of extrinsic regulation.

**Extrinsic cues promoting NPC maintenance and proliferation**

Among the various environmental regulators of cortical neurogenesis known to date, Notch signaling is one of the most well-studied. Several studies have shown that Notch signaling promotes NPC maintenance and self-renewal while repressing neurogenesis. Notch signaling is activated upon binding of transmembrane Notch ligands Delta or Jagged to Notch receptors (Notch1-4) on adjacent cells (Artavanis-Tsakonas et al., 1999; Yoon and Gaiano, 2005, Louvi and Artavanis-Tsakonas, 2006; Kopan and Ilagan, 2009; Ables et al., 2011). Upon binding and activation, the Notch intracellular domain (NICD) is cleaved by γ-secretase and translocates to the nucleus where it interacts with transcription factors such as RBP-J to activate *Hes1* and *Hes5* expression (Kageyama and Nakanishi, 1997). This signaling maintains RPs in the undifferentiated state (Gaiano et al., 2000; Yoon and Gaino, 2005). Moreover, Notch signaling is negatively regulated by the fate determinant Numb. Numb interacts with the NICD and targets it for degradation (McGill and McGlade, 2003; McGill et al., 2009). Numb’s distribution within RPs is regulated by the Par complex (Par3, Par6, aPKC). The latter localizes to the apical side of the RP where Par3 directly binds Numb, allowing for Numb phosphorylation and inhibition by aPKC (Goldstein and Macara, 2007; Nisihimura and Kaibuchi, 2007). This results in basal localization of Numb and its
asymmetric segregation into the differentiating daughter cell (Betschinger et al, 2003; Smith et al., 2007).

Fibroblast growth factors (FGFs) are also well-studied in the context of cortical neurogenesis. They are not only important regulators of regionalization, but play critical roles in NPC survival, proliferation, and differentiation. FGF ligands bind to tyrosine kinase receptors (FGFR1-4), which results in receptor dimerization, autophosphorylation, and activation, followed by induction of two intracellular cascades: the Ras/MEK/ERK mitogen activated protein kinase (MAPK) pathway and the phosphatidylinositol 3-kinase (PI-3K)/AKT pathway (Gauthier-Fisher and Miller, 2013). Both loss-of-function and gain-of-function studies show that FGF signaling promotes NPC proliferation and prevents premature differentiation (Dono et al., 1998; Vaccarino et al., 1999; Inglis-Broadgate et al., 2005). Of the 22 FGF family members, FGF2 is the most well-studied in the context of cortical neurogenesis. FGF2 acts as a mitogen for cultured NPCs (Rao, 1999; Temple and Qian, 1995; Vaccarino et al., 1999). Fgf2−/− mice have markedly reduced NPCs during early neurogenesis resulting in fewer neurons and glia in the mature brain (Dono et al., 1998; Ortega et al. 1998; Vaccarino et al., 1999; Korada et al., 2002; Raballo et al., 2000). FGF2 can also regulate astrogenesis in certain contexts. In cultured NPCs, FGF2 alone does not influence astrocyte formation, but can promote astrocyte formation in the presence of gliogenic cytokines such as ciliary neurotrophic factor (CNTF) (Qian et al., 1997; Song and Ghosh, 2004). In line with this, intraventricular injection of FGF2 into the E15/16 mouse brain increases RP proliferation without affecting astrocyte genesis, but the identical injection at later embryonic stages when the cytokine cardiotrophin-1 (CT-1) is expressed by projection neurons results in increased astrocyte formation (Vaccarino et al., 1999; Barnabe-
Heider et al., 2005). These observations highlight the fact that the response of an RP to a given growth factor depends in part on both the timing of exposure and the presence of other environmental cues. Other extrinsic factors that promote NPC maintenance and proliferation include epidermal growth factor (EGF), Wnt, and Shh. Conditional depletion of Shh signaling using an Emx1-Cre transgenic mouse resulted in reduced NPC proliferation, survival, and reduced IP and neuron formation (Komada et al., 2008). Interestingly, the response of NPCs to Shh is concentration dependent. In culture, lower Shh concentrations promote NPC proliferation while higher Shh concentrations have an inhibitory effect on proliferation (Palma and Ruiz I Altaba, 2004).

**Extrinsic cues promoting neurogenesis**

While Notch, FGF, EGF, Wnt, and Shh signaling positively regulate NPC maintenance and proliferation, other environmental signals actively promote differentiation of NPCs into neurons and glia. Platelet-derived growth factor (PDGF) and neurotrophins are two well-characterized growth factor families that promote neurogenesis. Neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) have been shown to regulate several aspects of nervous system development, including cortical neurogenesis (Kaplan and Miller, 2000). They bind to two receptors, the Trk tyrosine kinase receptor and the p75 neurotrophin receptor (P75NTR). BDNF and NT-3 are crucial for the survival of NPCs in culture, and knockdown of TrkB and TrkC via *in utero* electroporation results in decreased NPC proliferation and decreased neurogenesis (Barnabe-Heider and Miller, 2003; Bartkowska et al., 2007). Consistent with this, BDNF overexpression in RPs *in vivo* results in increased proliferation and neurogenesis (Bartkowska et al., 2007).
It is important to note that many of these ligands and receptors were discovered by analyzing single candidates independently using traditional assays. However, the complete picture of environmental signaling in the developing cortex is likely much more complex. A recent study used transcriptome and cell-surface proteomic analysis of cultured NPCs and newborn neurons of the developing cortex combined with mathematical modelling to systematically predict ligand-receptor interactions (Yuzwa et al., 2016). This systems-level analysis revealed a complex growth factor environment consisting of many previously uncharacterized autocrine and paracrine interactions. It also predicted several novel factors such as glial derived neurotrophic factor (GDNF), Neurturin (Nrtn), and interferon gamma (IFNγ), that were shown to promote neurogenesis in vivo (Yuzwa et al., 2016).

**Extrinsic cues regulating neuronal-subtype identity**

It is clear that many extrinsic cues converge on NPCs to determine the decision to self-renew or differentiate into a neuron (Yuzwa et al., 2016). Despite this knowledge, relatively little is known about how extrinsic cues regulate the genesis of particular neuronal subtypes. One extrinsic cue which is thought to play a role is the extracellular glycoprotein Reelin. Reelin is secreted by Cajal-Retzius cells in the marginal zone and acts as a migration cue for newborn neurons. In reeler mice (reelin deficient mice), newborn neurons fail to migrate past earlier born neurons, resulting in a failure of the preplate to split. As a result, these mutant cortices display highly-disorganized lamination (Kwan et al., 2012; Honda et al., 2011; Dekimoto et al., 2010; Boyle et al., 2011). One interpretation of these studies is that the correct positioning of neocortical neurons in the cortical plate (CP) may be important to ensure appropriate subtype identity. In line with this, recent work has suggested that the environment of a newborn neuron can influence its identity (Oishi et al., 2016). Knockdown
of Protocadherin20 (Pcdh20; normally expressed in post-migratory layer IV neurons) via *in utero* electroporation caused future layer IV neurons to inappropriately localize to layer II-III. Interestingly, these ectopically localized neurons (normally destined for layer IV) acquired layer II-III properties while losing their original layer IV properties (Oishi et al., 2016). The authors hypothesized that this positioning was important because layer IV neurons receive positional cues from thalamocortical axons (TCAs) which invade the cortex and project to layer IV neurons around the time layer IV neurons are maturing. In support of this hypothesis, they show that disruption of TCAs causes layer IV neurons to lose expression of upper-layer marker Rorb and to ectopic expression of deep-layer markers Lhx2 and Tbr1. These findings suggest that TCAs might provide cues to ensure appropriate specification of layer IV neurons (Oishi et al., 2016; Oishi and Nakajima, 2018). Together, these observations suggest that subtype specification can be influenced in part by extracellular signals acting on post-mitotic neurons. However, the ability of endogenous cues to regulate neuronal identity post-mitotically may be limited, as layer II/III neurons ectopically positioned in deep layer V do not acquire layer V properties (Ramos et al., 2006).

### 1.3 Post-transcriptional regulation of neurogenesis

Appropriate gene expression requires that proteins are produced at the correct time, amount, and subcellular location. This does not only require transcriptional control but further post-transcriptional regulatory mechanisms. Conventionally, mRNA concentration in mammalian cells was assumed to be highly concordant with protein concentration, and mRNA levels were thus commonly used as a proxy for protein levels. However, systems-
wide studies within the past decade have highlighted why this is not always a correct assumption. In this regard, numerous studies have found a moderate to low correlation between protein and mRNA levels ($R^2 \leq 0.4$), with translation rates contributing to a large percentage of the variance in protein levels (Gygi et al., 1999; Maier et al., 2009; Vogel et al., 2010; Schwanhausser et al., 2011; Avineri et al., 2013; Khan et al., 2013; Sharma et al., 2015). Other studies have challenged this conclusion, showing much higher correlations between mRNA and protein levels ($R^2 \sim 0.6-0.9$), suggesting that transcriptional control makes the largest contribution to protein levels (Vogel and Marcotte, 2012; Li et al., 2014; Jovanovic et al., 2015; Battle et al., 2015; Li and Biggin, 2015). Although transcriptional control is considered the main determinant of protein levels under steady state conditions, post-transcriptional control plays a dominant role during short-term state transitions such as differentiation (Hinnebusch and Natarajan, 2002; Beyer et al., 2004; Lu et al., 2009; Ingolia et al., 2011; Kristensen et al., 2013; Lee et al., 2013). Interestingly, transcription factors are particularly subject to post-transcriptional regulatory processes during these state transitions (Jovanovic et al., 2015). The ability to change mRNA “translation on demand” allows proteins to be rapidly available in response to extrinsic stimuli and cellular changes (Shim and Karin, 2002; Miller and Olivas, 2011; Liu et al., 2016). In addition to approximately 1600 transcription factors, there are over 1500 RNA-binding proteins (RBPs) encoded by the human genome (De Lima Morais et al., 2011; Castello et al., 2012). RBPs are highly ubiquitous post-transcriptional regulators that influence various steps of RNA metabolism including mRNA stabilization, degradation, localization, splicing, and translation (Keene, 2007). They interact with target mRNAs via RNA-binding domains (RBD) of various classes (Lunde et al., 2007). Both RBPs and microRNAs can assemble on mRNA sequences, often
located in 3’ untranslated regions (3’UTRs), to influence translation and/or decay (Duchaine and Fabian, 2018).

Translation initiation

Much post-transcriptional control involves the regulation of translation, particularly at the level of initiation. How is the translation of mRNAs initiated in mammalian cells? It begins with binding of the eIF4F eukaryotic initiation complex to the 5’ untranslated regions (5’UTRs) of mRNAs (Figure 4A). This complex is comprised of eIF4E, eIF4G, and eIF4A. eIF4E binds to the 5’-7-methylguanosine cap of the mRNA. eIF4G is a scaffold protein that bridges the 5’ and 3’ ends of mRNA via direct protein interactions with eIF4E at the 5’ end and poly (A) binding protein (PABP) at the 3’ poly (A) tail (Merrick and Pavitt, 2018; Hinnebusch and Lorsch, 2012). This allows for the formation of a “closed-loop” circular messenger ribonucleoprotein (mRNP) complex (Figure 4A). This activated complex is subsequently joined by the 43S preinitiation complex (PIC) consisting of the small 40S ribosomal subunit and the ternary complex of initiator methionyl-tRNA (Met-tRNAi) and GTP-bound form of eukaryotic initiation factor 2 (eIF2-GFP) (Hinnebusch and Lorsch, 2012). eIF4A, an RNA helicase, subsequently unwinds RNA in the 5’UTR allowing the 43S PIC to attach to mRNA and scan it for the initiation codon AUG. Recognition and base pairing of the PIC to the initiation codon triggers removal of the ternary complex from the 40S ribosomal subunit, and recruitment of the large 60S ribosomal subunit, forming the 80S initiation complex (IC). The IC consists of Met-tRNAi base-paired with the AUG start codon and is ready to begin actively translating the mRNA. This is followed by elongation and
termination steps of protein synthesis (Hinnebusch and Lorsch, 2012; Merrick and Pavitt, 2018).

**Translational control in mammalian stem cells**

One major lesson from studies in *Drosophila melanogaster* and *Caenorhabditis elegans* is that post-transcriptional regulatory mechanisms play critical roles in cell fate determination (Lasko, 2012; Nousch and Eckmann, 2013). Although translational control has been shown to be an important regulator of stem cell biology in these model organisms, much less is known about it in the mammalian context. However, recent work has shown that, similar to *D. Melanogaster* germline stem cells, low protein synthesis is a general feature of the stem cell state in several mammalian cell types including embryonic stem (ES) cells, hematopoietic stem cells, hair follicle stem cells, muscle stem cells, and neural stem cells (Sampath et al., 2008; Signer et al., 2014; Rodgers et al., 2014; Llorens-Bobadilla et al., 2015; Blanco et al., 2016; Sanchez et al., 2016; Zismanov et al., 2016; Teixera et al., 2018). In all these cases, the activation of protein synthesis promotes the onset of differentiation.

**Emerging role of post-transcriptional control in cortical neurogenesis**

As a rapidly evolving niche with many extrinsic cues interacting with intrinsic cellular programs, the developing neocortex is an ideal mammalian system to interrogate the role of post-transcriptional mechanisms. There are several advantages that post-transcriptional regulatory mechanisms provide over transcriptional control. Firstly, post-transcriptional control allows RPs to exist in a transcriptionally-primed state in which critical cell fate determinants regulating neurogenesis and specification are expressed at the mRNA, but not protein, level. These primed RPs can rapidly turn on/off gene expression in response to pro-
differentiation and/or pro-self-renewal cues. This ability is critical since RPs must undergo extensive rearrangements in morphology and begin migrating during differentiation.

Secondly, post-transcriptional control enables segregation of important fate determinants within asymmetrically dividing RPs. There is precedent for this in the developing neocortex; using a GFP-\textit{CyclinD2} 3’UTR reporter construct, \textit{CyclinD2} mRNA (a self-renewal factor) was shown to localize at the basal end feet of RPs where it is locally translated, a process that is dependent on transport elements within its 3’UTR (Tsunekawa et al., 2012;2014). During an asymmetric division, one daughter cell preferentially inherits the basal process and \textit{CyclinD2}, thus ensuring its sustained self-renewal capacity (Tsunekawa et al., 2012; 2014).

Thirdly, post-transcriptional control in the form of alternative splicing provides a way of vastly amplifying the protein diversity of the transcriptome by editing pre-mRNA sequences. In fact, the human brain expresses more alternatively spliced mRNA compared to other tissues, and alternative splicing is thought to largely contribute to the increased spatiotemporal complexity of the human brain (Yeo et al., 2004; Johnson et al., 2009). In addition to modifying pre-mRNAs to generate unique protein-coding transcripts, alternative splicing can modify the 5’ and 3’ UTRs of mRNAs to impact downstream elements of post-transcriptional processing including mRNA stability, export, and localization, by exposing or concealing binding sites for other RBPs and/or miRNAs (Pilaz and Silver, 2015). Several studies have shown dynamic alternative splicing changes in the developing forebrain across different brain regions, neuronal layers, and developmental stages (Johnson et al., 2009; Ayoub et al., 2011; Belgard et al., 2011; Dillman et al., 2013; Yan et al., 2015). One study, for instance, transcriptionally profiled cortical NPCs and neurons using RNA-sequencing and
showed that alternative exon usage during the NPC to neuron transition is mediated in part by splicing regulators Rbfox and Ptbp1, and is critical for neurogenesis (Zhang et al., 2016).

Although extensive work has been done to elucidate the role of epigenetic and transcription factors in cortical neurogenesis, the role of post-transcriptional control and RBPs in cortical development has only recently begun to be appreciated. In one study, DeBoer et al (2013) performed a microarray analysis at different timepoints throughout corticogenesis (at E11, E13, E15, and E18), specifically analyzing the expression of genes in post-transcriptional control associated gene ontology (GO) categories such as mRNA splicing, localization, decay, stability, translation, and RNA binding proteins (DeBoer et al., 2013). This revealed that post-transcriptional regulatory factors are dynamically expressed across the neurogenic period and that the greatest number of post-transcriptional control related genes that were differentially expressed were involved in translational control (DeBoer et al., 2013). A large number of RBPs were also dynamically expressed, and single-molecule fluorescence in situ hybridization (FISH) analyses of these RBPs found that their expression patterns were not only temporally dynamic but spatially specific in several cases (McKee et al., 2005; DeBoer et al., 2013).

To this date, only a small number of RBPs have been studied for their functional role in RBPs of the developing cortex. Due to space constraints, I will highlight only a few of these studies (for reviews on the topic, see DeBoer et al., 2013; Pilaz and Silver, 2015). One notable example is Human antigen R (HuR), a 3’UTR binding RBP that is a member of the Human Antigen (Hu) family of proteins. Polysome profiling showed that a large subset of transcripts are redistributed to different polysomal fractions upon conditional HuR knockout. These transcripts are enriched for regulators of transcription and translation, and are likely
functionally relevant, as P0 HuR conditional knockout cortices are thinner with defects in neuronal positioning and maturation (Kraushar et al., 2014). Musashi is another highly conserved RBP initially characterized in D. Melanogaster that plays a functional role in mammalian neurogenesis in culture (Sakakibara et al., 1996;1997;2001;2002). Mammals possess two Musashi homologs Musashi1 (Msi-1) and Musashi2 (Msi-2) which are expressed in adult and embryonic NSCs (Sakakibara et al., 1996;2001). NSCs dissociated from Msi-1−/− cortices and transfected with an antisense peptide against Msi-2 show reduced neurosphere formation and proliferative capacity (Sakakibara et al., 2002). This phenotype likely involves impaired cell-cycle progression, as Msi1/2 has been shown to regulate target mRNAs involved in differentiation and cell cycle progression such as the negative regulator of the Notch pathway Numb and cyclin-dependent kinase (CDK) inhibitor p21 (Imai et al., 2001; Battelli et al., 2006). Other RBPs shown to play important roles in the developing cortex include splicing regulators Magoh, Nova2, polypyrimidine-tract binding protein-2 (Ptbp2), and the regulator of mRNA stability and translation fragile X mental retardation protein (FMRP) (Silver et al., 2010; Yano et al., 2010; Licatalosi et al., 2012; Saffary and Xie, 2011; Li et al., 2014; Lennox et al., 2017).

Although this growing body of work has implicated translational control in the regulation of embryonic NPC proliferation and differentiation broadly, few studies have specifically addressed whether translation repression mechanisms regulate neuronal subtype specification. As discussed, most work in this regard has focused on transcriptional control (see section 1.2.2 and Figure 3). In my thesis, I have tested the hypothesis that translational repression mechanisms play important roles in regulating embryonic neuronal subtype
specification, focusing on two translational repressors, the eIF4E-T transporter (eIF4E-T/4E-T) and the RNA-binding protein Pumilio2 (Pum2).

1.3.1 4E-T

**Mechanism of repression**

Human 4E-T was first characterized as a nucleocytoplasmic shuttling protein that mediates the transport of eIF4E into the nucleus (Dostie et al., 2000). Despite this initial finding, the nuclear role of 4E-T remains poorly understood and most work to date has focused on its cytoplasmic role. The interaction of 4E-T and eIF4G with eIF4E is mutually exclusive; biochemical studies revealed that 4E-T interacts with eIF4E, but not eIF4G, via a conserved eIF4E recognition motif (YXXXXLH; H=hydrophobic amino acid, X=any amino acid) that is shared with eIF4G and 4E-binding proteins (4EBPs) (Mader et al., 1995; Dostie et al., 2000). Further studies showed that 4E-T colocalizes with eIF4E and mRNA decapping factors in P-bodies—cytoplasmic foci where mRNAs are stored, repressed and/or decayed—and represses cap-dependent translation without changing reporter mRNA levels (Ferraiuolo et al., 2005; Lee et al, 2008; Kamenska et al., 2014). These findings supported a model whereby 4E-T inhibits translation by competing with eIF4G for binding to eIF4E, thus inhibiting formation of the translation initiation complex (Figure 4B). Notably, 4E-T depletion also stabilizes ARE (AU-rich)-containing mRNAs and reduces silencing of miRNA-target mRNAs (Ferraiuolo et al., 2005; Kamenska et al., 2014). Consistent with these findings, tether function assays revealed that 4E-T can contribute to mRNA decay of specific mRNA subsets, suggesting a silencing mechanism that does not simply involve preventing the eIF4E-eIF4G interaction (Kamenska et al., 2014; Nishimura et al., 2015).
Using biochemical and structural approaches, two studies revealed that this mechanism may involve the interaction of 4E-T with the CCR4-NOT deadenylase complex and repressive decapping enzyme DDX6 (Ozgur et al., 2015; Nishimura et al., 2015). A model was proposed whereby 4E-T promotes mRNA decay by physically linking the decapping machinery (DCP1, DCP2) that assembles at the 3’ end along with CCR4-NOT, to the 5’ end of mRNAs, facilitating removal of the 5’-cap and mRNA decay by the 5’-3’ exonuclease XRN1 (Nishimura et al., 2015).

The *D. Melanogaster* protein Cup was initially and widely considered the functional homolog to h4E-T, similarly repressing cap-dependent translation by competing with eIF4G for eIF4E binding (Kamenska et al., 2014). However, there is limited sequence homology between them (13% identical residues and 22% similar residues) (Kamenska et al., 2014). Another protein, Dm4E-T, is closer in sequence (20% identical residues and 32% similar residues), and unlike Cup, forms P-bodies like human 4E-T when expressed in Hela cells (Kamenska et al., 2014). It is important to note that 4E-T homologs do not bind directly to mRNA and must be naturally tethered to them via sequence specific RBPs (Figure 4B). Cup, for instance, is recruited to the 3’UTR of mRNAs via interactions with RBPs Bruno and Smaug (Nelson et al., 2004).

**4E-T Function**

Insight into the biological functions of 4E-T come primarily from studies performed in flies, worms, and frogs. Via repression and localization of Nanos and Oskar mRNAs in early development, Cup regulates *D. Melanogaster* germ cell development (i.e. oogenesis from germ stem cell to mature egg), maintenance, and embryonic patterning (Nelson et al.,
The Xenopus homolog of 4E-T, X4E-T, interacts with RNA-binding protein CPEB to inhibit translation in early Xenopus oocytes and ensure proper oocyte development (Minshall et al., 2007; Standardt and Minshall, 2008). The C. elegans homolog of 4E-T IFET-1 also acts as a key regulator of germline development, repressing individual mRNAs via its interaction with RBPs OMA1/2 (Sengupta et al., 2013).

Is 4E-T mediated translational control similarly important in mammalian stem cells? Indirect evidence for a biological role in mammals came from a family-based genetic study that identified a heterozygous nonsense mutation in 4E-T associated with a dominant form of primary ovarian insufficiency (premature ovarian failure), suggesting a role in human ovarian germ cell development (Kasippillai et al., 2013). Our lab first identified a direct mammalian role for 4E-T in embryonic cortical neurogenesis (Yang et al., 2014). It was found that embryonic NPCs are transcriptionally primed to generate neurons, but that a translational repression complex containing the 4E-T protein maintains precursors in an undifferentiated state via repression of proneurogenic bHLH transcription factors such as Neurog1/2 and NeuroD. Consistent with earlier studies, 4E-T associated with P-body components Lsm1 and Ddx6 in cytoplasmic granules (Yang et al., 2014). A subsequent study showed that mammalian Smaug2 is one RBP that mediates the interaction between 4E-T and target mRNAs such as Nanos1 to maintain RPs in an undifferentiated state (Amadei et al., 2015).
Figure 4. Priming model of neurogenesis

(A) Translation initiation begins with binding of the eIF4F eukaryotic initiation complex to the 5’UTRs of mRNAs. This complex is comprised of eIF4E (orange), eIF4G (green), and eIF4A (maroon). eIF4E binds to the 5’-7-methylguanosine cap of the mRNA. eIF4G is a scaffold protein that bridges the 5’ and 3’ ends of mRNA via direct protein interactions with eIF4E at the 5’ end and poly (A) binding protein (blue) at the 3’ poly (A) tail. This allows for the formation of a “closed-loop” circular messenger ribonucleoprotein complex. (B) 4E-T (yellow) inhibits translation by competing with eIF4G for binding to eIF4E. 4E-T does not bind directly to mRNA and must be recruited via sequence specific RBPs (blue) (C) Embryonic NPCs are transcriptionally primed to make neurons, but a translational repression complex containing the 4E-T protein maintains NPCs in an undifferentiated state by keeping proneurogenic mRNAs in a translationally repressed state. In this model, neurogenic cues
would cause the disruption of this inhibitory complex, allowing the translation of proneurogenic proteins and the rapid initiation of neurogenesis (Yang et al., 2014).

**Regulation of 4E-T Activity**

Little is known about the extrinsic regulation of 4E-T activity. However, there is a small body of evidence suggesting that 4E-T, similar to other translational regulators, can be regulated by upstream signals. 4E-T is a phosphoprotein (Pyronnet et al., 2001; Dephoure et al., 2008), and oxidative stress triggers multisite phosphorylation (6 serine sites) of 4E-T by c-Jun-N-terminal kinase (JNK). Phosphorylation of 4E-T triggers its accumulation into larger P-bodies (Cargnello et al., 2012).

1.3.2 Pumilio2

PUF proteins are an evolutionary conserved family of RNA-binding proteins (RBPs) which act as key regulators of mRNA localization, stability, and translation (Miller and Olivas, 2011). They are conserved from fungi, molds, and parasites to flowering plants, moss, and metazoans (nematodes, amphibians, birds, fish and mammals). The first PUF protein, Pumilio, was discovered in *D. Melanogaster* and was shown to play a vital role in embryonic development (Nusslein-Volhard et al., 1987; Arvola et al., 2017). Specifically, Pumilio localizes and represses *hunchback* mRNA at the posterior pole of the *D. Melanogaster* embryo, ensuring that hunchback protein expression is restricted to the anterior region of the embryo. Pumilio mutant embryos have aberrant hunchback protein distribution and abdominal segmentation defects (Nusslein-Volhard et al., 1987; Arvola et al., 2017; Murata and Wharton, 1995).
Mammals have two classical Pumilio proteins, Pumilio1 (Pum1) and Pumilio2 (Pum2) that are closely related to one another (76% sequence identity) and to \textit{D. Melanogaster} Pumilio (30% sequence identity). They have high sequence specificity by virtue of a highly conserved RBD known as the Pumilio homology domain (Pum-HD) which has 80% sequence identity between humans and \textit{Drosophila} (Zamore et al., 1997). The Pum-HD consists of 8 alpha-helical repeats of an \textasciitilde{}36 amino acid long motif (Zamore et al., 1997; Wang et al., 2001).

\textbf{Mechanisms of Repression}

Pumilio proteins bind to a consensus binding site known as the Pumilio recognition/response element (PRE) \textasciitilde{}5'UGUAHAUA3' (H represents A, C, or U) (Zamore et al., 1997; Zamore et al., 1999, White et al., 2001, Wang et al., 2002, Hafner et al., 2010). Mammalian Pumilio proteins and their invertebrate orthologs repress target mRNAs containing PREs (Wickens et al, 2002; Miller and Olivas, 2011; Quenalt et al., 2011; Arvola et al., 2017). PREs are primarily located in the 3'UTRs of target mRNAs and less prevalent in the 5'UTR and coding sequences (Galgano et al., 2008; Bohn et al., 2018).

Pum1/2 proteins have been shown to repress target mRNAs in three main ways. One conserved mechanism from invertebrates to mammals is translational repression and mRNA decay via recruitment of the CCR4-NOT deadenylase complex, promoting deadenylation and decapping of target mRNAs (Van Etten et al., 2012, Goldstrohm et al., 2006, 2007; Blewett and Goldstrohm, 2012; Weidmann et al, 2014). Another conserved mechanism of repression involves antagonism of the poly(A) tail and translation activity of PABP. In this regard, the Pum-HD is thought to associate with PABP, disrupting PABP's contact with mRNA and its
ability to promote translation (Weidmann et al., 2014). Moreover, additional repression domains in the N-terminus of *D. Melanogaster* Pumilio can act autonomously to repress target mRNAs (Weidmann and Goldstrohm, 2012). Similar regions within the N-terminus of mammalian Pum1/2 have analogous repression activity (Weidmann and Goldstrohm, 2012).

A less well-characterized mechanism of mammalian Pum1/2 mediated repression involves the inhibition of translation elongation by inhibiting translation elongation factor eEF1A, a process dependent on Argonaute (Friend et al., 2012). Other mechanisms of repression involving interference with translation initiation have been identified in non-mammalian Pumilio orthologs. For instance, *D. Melanogaster* Pumilio can recruit mRNAs to the translational inhibitor 4EHP. 4EHP in turn prevents translation by competing with translation initiation factor eIF4E for binding to the mRNA cap (Cho et al., 2005). Similarly, *Xenopus* Pum2 can interfere with translation initiation by binding to the cap structure and interfering with binding of eIF4E (Cao et al., 2010).

More recent data suggests that mammalian Pum1/2 can activate certain mRNAs through currently unknown mechanisms. For instance, Pum1/2 can activate the expression of transcription factor *Foxp1* mRNA upon binding to PREs on its 3’UTR (Naudin et al., 2017). Activation of mRNAs by Pumilio proteins has also been demonstrated in multiple non-mammalian organisms and may involve mRNA stabilization, polyadenylation, and enhancement of translation (Pique et al., 2008; Archer et al., 2009; Suh et al., 2009; Kaye et al., 2009; Lee and Tu, 2015). This activation phenomenon seems to be transcript specific, suggesting that features of mRNAs are important.

**Pum1 and 2 Similarities and Differences**
Pum1 and Pum2 have RBDs that are 90% identical in sequence, and have been found to interact with largely overlapping sets of mRNAs. Moreover, they must be simultaneously depleted to fully alleviate the repression of PRE-containing reporter mRNAs (Wang et al., 2002; Galgano et al., 2008; Hafner et al., 2010; Zhang et al., 2017; Bohn et al., 2018). Pum1 and 2 proteins and mRNAs are expressed together in a large range of tissues and cell types throughout development (Consortium, 2013; Uhlen et al, 2015). Together, these observations suggest that they play largely overlapping roles. However, there is evidence that this is not always the case. One homolog is enriched over the other in certain tissues (Consortium, 2013). For instance, Pum2 is more highly expressed in blood and cerebellum compared with Pum1 (Consortium, 2013, Spassov and Jurecic, 2003). On the other hand, Pum1 is more highly expressed in skeletal muscle (Consortium, 2013). Moreover, Pum1 and Pum2 mutant phenotypes are distinct in certain contexts (see “Functions” section below).

**Pum1/2 target mRNAs**

Pumilio proteins can influence gene expression broadly. This is reflected by the fact that the presence of PREs are among the variables most highly correlated with mRNA instability (Yang et al., 2003; Schwanhausser et al., 2011). Earlier studies identified Pumilio interactions with individual genes, but more recent transcriptome wide analyses using approaches such as RIP-Chip and CLIP-Seq have identified extensive Pum1/2 regulatory networks (Bohn et al., 2018). Cumulatively, Pum1/2 have been shown to bind more than 4000 unique genes in human cells, mouse cells, and various tissues (Morris et al., 2008; Galgano et al., 2008; Hafner et al., 2010; Lee et al., 2016; Zhang et al., 2017). PREs are enriched in more than half of these mRNAs, suggesting that they are direct targets of Pum1/2 (Bohn et al., 2018). The remainder of mRNAs may be indirectly bound (i.e. another RBP
links Pum1/2 to target mRNAs), recognized via combinatorial mechanisms that alter the specificity of Pumilio, or simply false-positives.

Several lines of evidence suggest that Pum1/2 can interact with miRNA-mediated regulation. For instance, miRNA binding sites are enriched near PREs in several Pum1/2 targets (Galgano et al., 2008; Bohn et al., 2018). In addition, Pum1/2 interacts with Argonaute protein, a core component of the miRNA induced silencing complex (RISC) (Friend et al., 2012; Weidmann et al., 2014). Finally, Pum1/2 and PREs can enhance translational repression of $E2F3$ mRNA by miRNAs (Miles et al., 2012).

**Pum1/2 Function**

Pumilio proteins play many important roles in gametogenesis, embryogenesis, neuronal development, and neuronal function, and Pumilio dysfunction has been causally linked to neurological disorders and cancer (Wang et al., 2018; Goldstrohm et al., 2018). I will not cover all of these functions due to space constraints. Instead, I will focus on a few key functions that highlight the biological importance of Pum1/2. Pum1/2 have been shown to regulate stem cell activity in various contexts. For instance, in cultured ES cells, Pum1 reduces self-renewal and promotes differentiation in part by repressing transcription factors that mediate pluripotency (Leeb et al., 2014). Interestingly, Pum2 is not required in ES cells, suggesting that Pum1 and Pum2 can play divergent roles. Pum1/2 have also been shown to be necessary for the viability, growth, and development of mice. Double Pum1/2 knockout is embryonic lethal, while individual knockouts of either Pum1 or Pum2 results in reduced growth and size throughout life (Xu et al., 2007; Siemen et al., 2011; Chen et al., 2012; Mak et al., 2016; Zhang et al., 2017). Moreover, Pum1/2 play conserved roles in gametogenesis in...
both males and females. In *D. Melanogaster*, *C. elegans*, and mammals, disruption of Pum1/2 activity affects germline function and fertility (Zhang et al., 1997; Lin and Spradling, 1997; Forbes and Lehmann, 1998; Crittenden et al, 2002; Chen et al., 2012). For instance, male rodents with Pum2 gene trap mutations have smaller testes and deteriorated seminiferous tubules (Chen et al., 2012, Xu et al., 2007).

Pumilio proteins play important conserved roles in developing and mature neurons. Mammalian Pum2, for instance, regulates dendritic morphology and electrophysiology of hippocampal neurons by regulating mRNAs encoding the voltage gated sodium channels *Nav1.1* and *Nav1.6* (Vessey et al., 2006; Vessey et al., 2010; Driscoll et al., 2013). Disruption of Pum2 results in increased neuronal excitatory current frequency, and loss of Pum2 in mice results in an increased predisposition to seizures (Vessey et al., 2010; Follwaczny et al., 2017). Pumilio proteins also play important roles in neuronal differentiation. In this regard, two independent studies showed that mammalian Pum2 is involved in the asymmetric segregation of cell fate determinants during cortical neurogenesis (Vessey et al., 2012; Kusek et al., 2012). Specifically, the authors showed that a complex containing the RBPs Staufen2 and Pum2 interacts with proneurogenic mRNAs such as *Prox1* in NPCs. During a neurogenic division, this complex preferentially segregates *Prox1* to Tbr2-positive IPs. Disruption of the complex causes mislocalization and misexpression of *Prox1*, and premature differentiation (Vessey et al., 2012; Kusek et al., 2012). Consistent with the important role of Pumilio proteins in NPCs and post-mitotic neurons, Pumilio loss of function mutations have been linked to neurological and neurodevelopmental disorders characterized by intellectual disability, developmental delay, seizures, and ataxia (Gennarino et al., 2018).
**Subcellular Localization**

Pum1/2 localize in the cytoplasm and are observed in three types of cytoplasmic ribonucleoprotein (RNP) granules: stress granules, P-bodies, and neuronal transport granules. Pum1/2 proteins have been observed in stress granules that form in response to oxidative stress and viral infection (Narita et al., 2014; Khong et al., 2017). Pum1/2 are also enriched in purified P-bodies (Hubstenberger et al., 2017). Moreover, Pum1/2 are found in granules that are transported within neuronal dendrites, where they act to deliver mRNA to synapses to allow for local translation in response to synaptic activity (Vessey et al., 2010; Vessey et al., 2006).

**Regulation of Pum1/2 Activity**

Pum1/2 proteins are subject to various forms of regulation. Pum1 and Pum2 have 8 and 13 PREs within their 3’UTRs respectively, enabling them to autoregulate their own expression. In fact, Pum1/2 binding to their own and each other’s mRNAs has been observed in various cell types (Morris et al., 2008; Galgano et al., 2008; Zhang et al., 2017). Consistent with this finding, depletion of one Pumilio homolog can lead to the increased expression of the other (Kedde et al., 2010; Zhang et al., 2017). In addition to auto- and cross-regulation, Pum1/2 expression can be regulated via miRNA-mediated mechanisms. For instance, the 3’UTR of Pum2 homologs in humans, mice and rats contain miR-134 binding sites, and miR-134 mediated repression of Pum2 promotes dendritic outgrowth of rat hippocampal neurons (Fiore et al., 2009).

Pum1/2 can act in a combinatorial fashion with other RBPs, which can alter its regulation and target specificity (Pique et al., 2008; Campbell et al., 2012; Weidmann et al.,...
2016; Arvola et al., 2017). The requirement for these binding partners is often organism and transcript dependent. For instance, Pumilio-mediated repression of hunchback mRNA in D. Melanogaster is dependent on the formation of a quaternary complex that contains Brain tumour (Brat), Nanos, Pumilio, and mRNA (Wreden et al., 1997; Sonoda and Wharton, 1999; Sonoda and Wharton, 2001), while binding to the mRNA cyclin B requires Nanos but not Brat (Kadyrova et al., 2007). These binding partners directly influence the target specificity of Pumilio proteins. This was neatly demonstrated using a yeast three hybrid assay, where Pum2 and the RBP Deleted in Azoospermia-Like (DazL) cannot stably interact with SDAD1 3’UTR, while Pum2 and BOULE (BOL) can (Urano et al., 2005). Several of these interactions are conserved in mammals, as orthologs of Nanos (Nanos1,2,3), Brain Tumour (Trim71) and CPEB (Jaruzelska et al., 2008; Campbell et al., 2012; Loedige et al., 2013) have been shown to interact with mammalian Pum1/2. These protein-protein interactions can either be RNA-dependent or independent depending on the cellular context (Sonoda and Wharton, 1999; Nakahata et al., 2001; Jaruzelska et al., 2008).

Pumilio proteins are also subject to post-translational modifications that can alter their activity. Several phosphorylation, ubiquitylation, and methylation sites have been mapped to Pum1/2 (Goldstrohm et al. 2018). Phosphorylation of yeast Puf6p by protein kinase CK2 relieves its repression of Ash1 mRNA, and phosphorylation of mammalian Pum1 on S714 is induced by growth factors and promotes its interaction with and repression of p27 mRNA (Kedde et al., 2010).
1.4 Postnatal and adult V-SVZ neurogenesis

It was originally believed that neurogenesis did not persist beyond embryonic development into adulthood. This dogma was first challenged in 1962 by thymidine-H\(^3\) labelling experiments which revealed newborn neurons in the adult rat cortex following electrolytic lesion (Altman, 1962). This was followed by several other observations showing newborn neurons in multiple brain regions of adult rats and cats including the hippocampus, cerebral cortex, and cerebellum under normal conditions (Altman, 1963; Altman and Das, 1965). Since then, extensive work has been carried out to characterize postnatal and adult neurogenesis, particularly in rodents.

During postnatal and adult life, new neurons are generated from populations of stem and progenitor cells collectively termed neural precursor cells (NPCs). There are two major niches in which neurogenesis occurs in the postnatal brain: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the ventricular-subventricular zone (V-SVZ) surrounding the lateral ventricles (Figure 5). I will focus on V-SVZ neurogenesis, as it is most relevant to my thesis.

1.4.1 Cellular composition of the V-SVZ

Postnatal and adult NSCs reside within an epithelium known as the V-SVZ that is located directly adjacent to the lateral ventricles of the brain (Figure 5A) (Doetsch et al., 1999, 2002; Conover et al, 2000; Mirzadeh et al., 2008). It contains multiple neural precursor populations including NSCs (also known as B1 cells), transit-amplifying (TA) cells (or type C cells), and neuroblasts (or type A cells) (Figure 5A,B). V-SVZ NSCs give rise to TA cells, which divide symmetrically before generating neuroblasts (Doetsch et al., 1999; Ponti et al.,
2013) (Figure 5B). These neuroblasts migrate along the rostral migratory stream (RMS) to the olfactory bulb (OB), where they differentiate into various subtypes of interneurons (Ponti et al., 2013; Bond et al., 2015; Lim and Buylla, 2014; 2016). These newborn interneurons integrate into the existing OB circuitry and influence the plasticity of olfactory-related behaviors such as social/mating recognition (pheromone related behaviors) and olfactory memory (Ming and Song., 2011). Moreover, NSCs can also give rise to oligodendrocytes in both healthy and demyelinating conditions (Menn et al., 2006; Xing et al., 2014), and astrocytes after ischemic cortical injury (Benner et al., 2013).

Several studies have suggested that that NPC number, proliferation, and new neuron formation, declines with age (Luo et al., 2006; Ahlenius et al., 2009; Ben Abdallah et al., 2010; Shook et al., 2012; Capilla-Gonzalez et al., 2014; Daynac et al., 2016). Consistent with a decrease in the production of new OB neurons, aged mice exhibit deficits in scent discrimination compared to younger mice (Enwere et al. 2004). Despite this, there is still debate regarding the extent of NSC decline with age.
Figure 5. The postnatal/adult ventricular-subventricular zone niche.

(A) Lining the lateral ventricles of the postnatal brain is a cell dense area known as the V-SVZ. The V-SVZ contains several cell types: ependymal cells (brown), neural stem cells (blue), transit amplifying cells (red), and neuroblasts (orange). (B) V-SVZ NSCs give rise to TA cells before generating neuroblasts. These neuroblasts divide one to two times as they migrate towards the olfactory bulb, where they differentiate into various subtypes of interneurons. Alternatively, NSCs can also give rise to oligodendrocytes. (C) Different
subpopulations of embryonic RPs give rise to distinct V-SVZ NSC compartments postnatally. Dorsal embryonic RPs contribute to NSCs residing in the dorsal and dorsolateral corner of the postnatal V-SVZ (green). Ventral embryonic RPs from the ganglionic eminence contribute to the NSCs residing in the lateral wall of the postnatal V-SVZ (blue). Septal RPs contribute to NSCs residing in the medial wall of the postnatal V-SVZ (purple). CC=corpus callosum; LV= lateral ventricle.

**Precursors: V-SVZ Neural Stem Cells**

Adult NSCs express several glial markers such as glial-fibrillary acidic protein (GFAP), glutamate aspartate transporter (GLAST), and brain lipid-binding protein (BLBP), as well as precursor-specific markers such as Sox2 and the intermediate filament Nestin (Zhang and Jiao, 2015). They possess a radial morphology, extending a thin apical process that intercalates between ependymal cells to contact the ventricle and a basal process that terminates on blood vessels (Doetsch et al., 1999; 2002; Conover et al., 2000; Mirzadeh et al., 2008) (Figure 5A).

Adult NSCs possess the cardinal properties of somatic stem cells: self-renewal and multipotency. The self-renewal capacity of NSCs has been shown both by in vivo clonal lineage tracing approaches and in vitro time-lapse imaging (Ortega et al., 2013; Obernier et al., 2018). Furthermore, they are multipotent, not only capable of giving rise to inhibitory interneurons, but also astrocytes and oligodendrocytes. Retroviral fate mapping of V-SVZ NSCs showed that in uninjured mice, NSCs can generate oligodendrocytes destined for the corpus callosum where they function to myelinate axons (Menn et al., 2006; Xing et al., 2014). Consistent with this finding, single GFAP-positive cells isolated from the V-SVZ via FACS and cultured on astrocyte monolayers were shown to give rise to both neuronal and glial lineages (Menn et al., 2006). The authors also demonstrate that oligodendrocyte output
increases after demyelinating injury, suggesting that NSCs can respond to environmental cues. In contrast to these findings, other in vitro time lapse imaging studies have shown that individually isolated NSCs cultured in growth-factor free media can generate either neurons or oligodendrocytes, but not both (Ortega et al., 2013). In addition to oligodendrocytes, V-SVZ NSCs can generate astrocytes in an injury context (Benner et al., 2013). Using a NestinCreERT2 transgenic line, the authors showed that NSCs can generate astrocytes that migrate to the injury site following a photothrombotic ischemic cortical injury (Benner et al., 2013). Together, these studies argue that extrinsic niche signals are important regulators of NSC fate choices.

**Transit Amplifying Cells**

TA, or type C cells, are produced directly from NSCs, and also express the precursor markers Sox2 and Nestin. They are often distinguished from other postnatal and adult NPCs by their protein expression of the bHLH transcription factor Ascl1/Mash1. These Ascl1-positive precursors have been shown to generate both neuronal and oligodendrocyte lineages (Parras et al., 2004). Akin to embryonic IPs, TA cells act to increase the pool of mitotically active precursors and increase the output of differentiated progeny. These cells have limited self-renewal capacity, dividing symmetrically (three times on average) to generate more restricted neuroblasts (Ponti et al., 2013).

**Neuroblasts**

Neuroblasts, or type A cells, are the neuronally-biased progeny of TA cells. In contrast to TA cells, they express the microtubule associated protein doublecortin (Dcx) and cell-surface molecule polysialyalted neural-cell-adhesion molecule (PSA-NCAM) (Doetsch
et al., 1997). Dcx and NCAM are both important for neuronal migration embryonically (Tomasiewicz et al., 1993, Cremer et al., 1994; Ono et al., 1994; Hu et al., 1996; Francis et al., 1999). Like TA cells, neuroblasts can divide symmetrically, but their self-renewal capacity is more limited (Ponti et al., 2013). Unlike other precursors, these neuroblasts have extensive migratory capacity. In *vitro*, neuroblasts can form chains of themselves and migrate at speeds of 120 micrometers per hour (Wichterle et al 1997). They possess a characteristic bipolar morphology with a leading process which extends in the direction of migration and a smaller trailing process (Petreanu and Alvarez-Buylla, 2002; Brown et al., 2003). *In vivo*, these neuroblasts migrate away from the V-SVZ niche towards the OB through the RMS. Once they reach the OB around two weeks after their birth, neuroblasts migrate radially away from the RMS and complete their differentiation into interneurons (Petreanu and Alvarez-Buylla, 2002).

### 1.4.2 Radial precursor cell origin of V-SVZ NSCs

Postnatal and adult V-SVZ NSCs preserve key features of embryonic RPs (see section 1.1.1). They express Nestin, BLBP, GLAST, and Sox2 (Lagace et al., 2007; Giachino et al., 2014), possess prominent apical and basal processes (Mirzadeh et al., 2008), and are located adjacent to the lateral ventricles, with which they make contact. These molecular, spatial, and morphological similarities suggested that V-SVZ NSCs may be related to embryonic RPs. The first direct link between V-SVZ NSCs and embryonic RPs came from studies in which RPs were selectively labeled with an adenovirus-carrying Cre-recombinase inducing recombination of reporter genes in subsets of RPs (Merkle et al., 2004). Using this approach, the authors showed that V-SVZ NSCs, which give rise to inhibitory interneurons and glia postnatally, are derived from embryonic RPs (Merkle et al., 2004). A model was
thus proposed whereby RPs transition to postnatal V-SVZ NSCs after the generation of excitatory neurons and glia embryonically is complete (Kriegstein and Buylla, 2009).

However, this model was recently challenged by two studies. In one study, the authors labelled embryonic NPCs with GFP-containing, barcoded retroviruses at different embryonic ages, and adult NSCs were subsequently labelled with tdTomato. This analysis revealed that adult V-SVZ NSCs are derived from embryonic RPs that divide prior to E15.5 and remain relatively quiescent until they begin generating interneurons postnatally (Golden et al., 1995; Fuentealba et al., 2015). Consistent with this finding, another study analyzed the retention of a GFP-tagged histone 2B (H2B) fusion protein in dividing embryonic NPCs and showed that adult NSCs are derived from a subpopulation of GFP-label retaining, slowly-dividing NPCs that emerges between E13-E15 (Furutachi et al., 2015). These findings suggest that the lineage relationship between RPs and V-SVZ NSCs is not linear as early models proposed, but that adult and embryonic lineages bifurcate during mid-embryonic development. A recent single-cell RNA sequencing study supports this model, showing that RPs transition to a non-proliferative state during embryogenesis and that this population shares transcriptional similarities with adult V-SVZ NSCs (Yuzwa et al., 2017).

Several lineage tracing studies have now shown that distinct subpopulations of embryonic RPs give rise to different postnatal V-SVZ NSC compartments (Figure 5C). For instance, dorsal embryonic RPs which generate cortical excitatory neurons embryonically contribute to NSCs residing in the dorsal and dorsolateral corner of the V-SVZ (Figure 5C). In contrast, ventral embryonic RPs from the ganglionic eminence (GE) contribute to NSCs residing in the lateral wall of the V-SVZ (Figure 5C). In fact, the vast majority (~70%) of adult NSCs originate from these ventral RPs (Young et al., 2007; Kohwi et al., 2007;
Gallagher et al., 2013; Fuentealba et al., 2015). In light of this relationship between embryonic RPs and adult NSCs, it is perhaps less surprising that perturbations during embryonic neurogenesis result in long-term alterations in adult V-SVZ NSCs and neurogenesis (Gallagher et al., 2013; Yang et al., 2016; Hu et al., 2017).

1.4.3 Intrinsic/extrinsic regulation of V-SVZ NSCs

Transcriptional and epigenetic regulation

Similar to embryonic RPs, the precise regulation of postnatal NSCs is dependent on the interplay between intrinsic factors and extrinsic cues. Transcriptional control is a fundamental intrinsic regulator of postnatal NSC biology, and several transcription factors have been identified as important for the regulation of NSC maintenance, differentiation, and cell fate determination (i.e. neuronal versus glial fate commitment). Once a cell is biased towards the GABAergic interneuron lineage, additional transcription factors regulate subsequent subtype specification. However, I will not be discussing these downstream factors as they are beyond the scope of my thesis.

One well studied transcription factor that plays an important role in NSC maintenance is SRY (sex determining region Y)-box 2 (Sox2) (Ellis et al., 2004). Sox2 is highly expressed in multiple cell types within the V-SVZ including NSCs, and its expression requires Notch signaling. Sox2 deletion leads to NSC depletion and impaired adult V-SVZ neurogenesis (Ferri et al., 2004). Sox2 can also be modulated by interactions with other transcriptional co-factors and by posttranslational modifications (Sarkar and Hochedilinger, 2013). For instance, Sox2 expression in NSCs is activated by another transcription factor Arsenite-
resistance protein 2 (Ars2), and deletion of Ars2 results in loss of NSCs, a phenotype that can be rescued by Sox2 overexpression (Andreu-Agullo et al., 2012).

Other transcription factors regulate the decision of a postnatal NSC to become biased towards a neuronal and/or glial lineage. A prototypical example is the bHLH transcription factor Ascl1/Mash1, which is expressed primarily in TA cells (Parras et al., 2004; Kim et al., 2011). Postnatal ablation of Ascl1 results in impaired V-SVZ OB neurogenesis and oligodendrogenesis, suggesting that Ascl1 is required for the specification of both neurons and glia (Parras et al., 2004). Olig2, another bHLH transcription factor, is expressed in a subset of V-SVZ NSCs and TA cells, and overexpression of Olig2 promotes oligodendrocyte genesis and represses neurogenesis (Hack et al., 2005). Consistent with this finding, expression of a dominant negative form of Olig2 inhibits oligodendrocyte production and induces ectopic neuronal marker expression (Marshall et al 2005). Furthermore, the transcription factor Pax6 promotes the differentiation of olfactory bulb periglomerular interneurons (Kohwi et al., 2005; Hack et al., 2005). Pax6 and Olig2 show a mutually exclusive protein expression pattern in the neonatal V-SVZ, and retroviral overexpression of Pax6 in the neonatal V-SVZ downregulates Olig2 to promote a neurogenic fate at the expense of gliogenesis (Hack et al., 2005; Jang and Goldman, 2011). Moreover, the transcription factors Sp8 and Sp9 (expressed in neuroblasts, immature, and mature interneurons) are necessary for survival, migration, and differentiation of olfactory bulb interneurons (Waclaw et al., 2006; Li et al., 2017). Similarly, Dlx1 and Dlx2 (expressed in TA cells and neuroblasts) are necessary for the genesis of olfactory bulb interneurons (Doetsch et al., 2002; Brill et al., 2008).
Epigenetic mechanisms that alter chromatin state to either activate or repress the transcription of genes also plays important regulatory roles postnatally. For instance, histone deacetylases (HDACs) are epigenetic modifiers that play key roles in regulating V-SVZ neurogenesis. Administration of HDAC inhibitors to postnatal mice results in disruption of V-SVZ neurogenesis (Foti et al., 2013). Moreover, HDAC2 deletion in V-SVZ NSCs leads to reduced neurogenesis (Jawerka et al., 2010). Several epigenetic modifiers have been shown to interact with important transcription factors such as Pax6 to execute their function (Ninkovic et al., 2013).

**Beyond transcriptional and epigenetic control**

Emerging evidence indicates that additional layers of gene expression control are important within NSCs. A recent single cell RNA sequencing study of adult NSCs revealed transcriptional upregulation of ribosomal genes and increased protein synthesis as NSCs transition from a dormant/quiescent state, to an activated/dividing state (Llorens-Bobadilla et al., 2015). In line with these findings, mTORC1, an inducer of global protein synthesis, is activated when NSCs transition to TA cells (Paliouras et al., 2012; Mahoney et al., 2016), and inhibiting mTORC1 activity prevents neonatal NSC differentiation and depletes the TA pool (Paliouras et al., 2012; Hartman et al, 2013). In addition to the regulation of global translation, there is evidence of translational control of specific cell fate regulators. microRNAs have been shown to regulate V-SVZ NSC self-renewal and neuronal differentiation by binding to the UTRs of target mRNAs. For example, miR-124 promotes cell-cycle exit and neuronal differentiation by suppressing Sox9 mRNA (Cheng et al., 2009). Other miRNAs, such as miR-137, have been shown to inhibit differentiation and promote NSC proliferation (Szulwach et al., 2010). Interestingly, it was found that Pax6 protein
expression is restricted to the dorsal wall of the V-SVZ, while Pax6 mRNA is expressed all along the dorsal-ventral axis. This restricted expression pattern was due to miR-7a mediated translational repression of Pax6 mRNA in ventral regions (De Chevigny et al, 2012). Other regulators of gene expression include long non-coding RNAs, which are beyond the scope of the thesis.

**Extrinsic cues**

V-SVZ NSCs reside in a specialized niche rich in extrinsic cues that regulate their proliferation, differentiation, and cell fate choices. These include signaling molecules also critical for patterning and specification embryonically including SHH, Wnt, retinoic acid, Notch, BMPs and Ephrins (for an extensive review on the topic, see Ihrie and Alvarez-Buylla 2011). Some cues regulate neurogenic versus gliogenic fate commitment. BMP signaling, for instance, promotes gliogenesis at the expense of neurogenesis (Lim et al., 2000). Many of the extrinsic cues which converge on NSCs originate from surrounding cell types such as ependymal cells, vascular cells, astrocytes, and more committed progeny of NSCs. For instance, endothelial cells secrete vascular endothelial growth factor (VEGF) which promotes neurogenesis (Jin et al 2002). They also secrete betacellulin, an EGF like growth factor, which positively regulates NSC proliferation and neuroblast production (Gomez-Gaviro et al., 2012). Neurotransmitters also feed onto NSCs to regulate their behavior. For instance, NSC progeny such as neuroblasts can secrete GABA, which acts upon GABA-A receptors on NSCs to inhibit cell proliferation and neuronal differentiation. This functions as a negative feedback mechanism to downregulate their own production (Liu et al 2005). Circulating systemic factors also regulate NSC biology. This was highlighted by a study which showed that the blood of young animals "rejuvenate" the older neurogenic niche by increasing both
NSC number and neurogenesis. (Katsminpardi et al., 2014). By virtue of their apical contacts with the ventricles, NSCs are also regulated by CSF factors such as IGF-2, which have been shown to regulate the proliferation of NSCs (Lehtinen et al., 2011).

In summary, postnatal and adult V-SVZ NSCs, like their embryonic predecessors, are extensively regulated by several extrinsic cues which cooperate to influence NSC maintenance, differentiation, and cell fate commitment. This raises the interesting question of how NSCs integrate all these factors in a rapid fashion to execute a particular decision.
Chapter 2. Rationale & Hypothesis

The diverse types of neurons that are organized into layers in the mammalian cerebral cortex are the fundamental requirement for the assembly of complex circuitry. However, the mechanisms regulating the genesis of these distinct neuronal populations from embryonic NPCs are still not well understood. Although extensive work has been done to address how this process unfolds, the most well-studied mechanisms of neuronal subtype specification have focused on transcriptional control (Leone et al., 2008; Greig et al., 2013). It is unclear, however, whether translational regulatory mechanisms play a similarly important role in the developing forebrain. Therefore, the overarching hypothesis of this thesis is that translational control plays an essential role in neuronal differentiation and fate specification in the developing forebrain.

Previous work has suggested that translational repression mechanisms play a role in regulating neuronal differentiation during embryonic cortical development. In this regard, our lab previously demonstrated that embryonic NPCs are transcriptionally primed to generate neurons, but that a translational repression complex containing the 4E-T repressor maintains them in an undifferentiated state by keeping mRNAs encoding proneurogenic factors in a translationally silenced state (Amadei et al., 2015; Yang et al., 2014). To follow up on these observations, I asked whether translational repression mechanisms played other roles in cortical development. To do so, I revisited a list of mRNAs which interact with 4E-T in the embryonic cortex from a previously published 4E-T RNA-immunoprecipitation analysis (Yang et al., 2014). Interestingly, I observed mRNAs that encode transcription factors important for the specification of unique neuronal subtypes. These observations led us to
hypothesize that translational control plays a role in regulating neuronal subtype specification in the embryonic cortex. To test this hypothesis, the experiments in Chapter 4 were performed. In this chapter, I provide evidence that embryonic cortical radial precursors co-express mRNAs encoding specifiers for multiple cortical neuron subtypes, and that a Pum2-4E-T complex represses the translation of some of these mRNAs to ensure the appropriate specification of daughter neurons.

Although it is well-established that a subpopulation of embryonic NPCs persists postnatally and into adulthood within the ventricular-subventricular zone (V-SVZ), whether translational repression continues to play a role in regulating cell fate decisions postnatally remains largely unknown (Fuentealba et al., 2015; Furutachi et al., 2015). Based on my previous work, I hypothesized that 4E-T mediated translational control is a critical regulator of neural precursor maintenance and differentiation in the postnatal brain. To test this hypothesis, I conditionally knocked out 4E-T in NPCs postnatally using a mouse we have generated carrying a floxed allele of the 4E-T locus. These experiments are described in Chapter 5. In this chapter, I provide evidence that 4E-T continues to act as a critical regulator of NPC maintenance and differentiation postnatally, akin to its embryonic role.

Taken together, the work presented in this thesis challenges existing transcriptional models to show that neuronal differentiation and subtype specification is controlled by translational mechanisms. It also raises important questions apt for future investigation. What is the entire spectrum of post-transcriptional machinery operating in embryonic and postnatal NPCs? What are the specific targets of these regulators, and to what extent do they overlap? What are the environmental cues that feed into these repressive complexes to trigger their
association and/or dissociation? Is transcriptional flexibility and translational repression a general cellular strategy employed in other developing mammalian stem cell niches? Future work should focus on whether translational mechanisms regulate cell fate specification postnatally, and how these pathways are altered in the context of neuronal injury and neurodevelopmental disorders.
Chapter 3. Materials & Methods

3.1 Animals and injections

All animal use was approved by the Animal Care Committee of the Hospital for Sick Children in accordance with the Canadian Council of Animal Care policies. Mice were maintained on a 12hr light/dark cycle, and food and water was provided ad libitum. All mice were healthy with no obvious behavioral phenotypes, and none of the experimental mice were immune compromised. For all studies, mice of either sex were used and mice were randomly allocated to experimental groups. Embryonic (E) day 11-17 and postnatal (P) day 3 mice were used. Wild type CD1 mice (Charles River Laboratories) were used for all culture and electroporation experiments unless otherwise indicated.

The 4E-T floxed mice were generated from cryopreserved mutant C57BL/6 ES cells purchased from the Knockout Mouse Project (KOMP) under the International Knockout Mouse Consortium. These ES cells have a 4E-T allele containing a lacZ trapping cassette (knockout-first allele) (Skarnes et al., 2011). Mutant ES cells were used to generate chimeric mice, which were subsequently crossed until the modified ES cells integrated into the germline. Mice that were heterozygous for the knockout-first 4E-T allele were subsequently crossed to mice expressing the flippase (FLP) recombinase (C57/BL/6-Tg(Pgk1-flpo)10Sykr/J) (Wu et al., 2009). Flp-mediated recombination converted the knockout-first allele into a conditional allele containing loxP sites flanking critical exon 4 of 4E-T (4E-T\(^{0/-}\)). 4E-T\(^{0/-}\) mice were backcrossed to wild type (4E-T\(^{v/v}\)) mice for several generations to remove the FLP recombinase, and finally crossed to one another to generate 4E-T\(^{0/0}\) homozygotes.
All information about this allele can be found on the following website:

http://www.mousephenotype.org/data/alleles/MGI:1921453/tm2a(KOMP)Wtsi. The following primers were used for genotyping: CSD-F (AGCCCAGTATGGTGCTGCATGC); CSD-ttR (CAAATGAAACTGACAGTCCAGAACTCC); CAS_R1_Term (TCGTGGTATCGTTATGC GCC).

4E-T genotyping parameters:

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Nestin-CreERT2 (C57BL/6-Tg(Nes-cre/ERT2) (Imayoshi et al., 2006) and WT C57BL/6J mice were obtained from The Jackson Laboratory. For perinatal 4E-T ablation, 1 day following birth, lactating females were injected i.p. with 1 mg tamoxifen in sunflower oil, twice daily for 3 days (age of littermates: P1-P3). Littermates were injected i.p. with 100 mg/kg BrdU once at P7, and analyzed the next day at P8. All mice were bred and genotyped as recommended by The Jackson Laboratory.
3.2 Primary cell cultures and transfections

Primary cell cultures were prepared as previously described (Yang et al., 2014). Briefly, cortices were dissected from pooled E11-E13 CD1 mouse embryos of either sex from the same mother. The meninges were removed and the exposed cortex was collected and mechanically triturated. Dissociated cortical precursor cells were cultured at 37°C in Neurobasal medium (Invitrogen) supplemented with 2% B27 (Invitrogen), 0.5 mM L-glutamine (Invitrogen) and 40 ng/ml FGF2 (BD Biosciences), at a density of 300,000 cells/ml on glass coverslips precoated with 2% laminin (BD Biosciences) and 1% poly-D-lysine (Sigma), and transfected with Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. For co-transfection, a 1:3 ratio of EGFP to shRNA (total 1 μg/well) was used. Cells were collected and immunostained 48hr or 72hrs later.

3.3 Plasmids

The pEF-EGFP plasmid expressing nuclear EGFP (Barnabé-Heider et al., 2005), the pcDNA3.1(-) plasmid expressing the CBP phosphomimic (Wang et al., 2010; 2012), and the shRNAs against 4E-T (Yang et al., 2014) or Pumilio2 (Vessey et al., 2012) have been previously described.

3.4 In utero electroporation

CD1 timed pregnant mice were used for in utero electroporations as previously described (Gauthier et al., 2007). Briefly, an expression construct for nuclear EGFP was coelectroporated with shRNA constructs at a 1:3 ratio. Prior to injection, plasmids were mixed with 0.5% trypan blue. Following injection into the lateral ventricles, the square
electroporator CUY21 EDIT (TR Tech, Japan) was used to deliver five 50 ms pulses of 40-
50 V with 950 ms intervals per embryo. Brains were dissected 48hr or 72hrs later and
analyzed post electroporation at indicated developmental stages.

3.5 Antibodies

The primary antibodies used were mouse anti-4E-T (Novus Biologicals, 1:500), chicken anti-
GFP (Abcam, 1:2000, RRID:AB_300798), mouse anti-Ki67 (BD Biosciences Pharmingen,
1:500, RRID: AB_396287), mouse anti-βIII-tubulin (Biolegend, 1:1000,
RRID:AB_10063408), rabbit anti-βIII-tubulin (Biolegend, 1:1000, RRID: AB_2564645),
rabbit anti-Pax6 (Biolegend, 1:2000, RRID: AB_2565003), rabbit anti-Tbr2 (Abcam, 1:500,
RRID: AB_778267), rat anti-Ctip2 (Abcam, 1:200, RRID: AB_2064130), rabbit anti-Tle4
(gift from Stefano Stifani, 1:500), rabbit anti-Pumilio2 (MBL, 1:1000, RRID: AB_1953053),
rabbit anti-Pumilio2 (Bethyl Laboratories, 1:500, RRID: AB_2173752), goat anti-Brn1/2
(Santa Cruz Biotechnology, 1:250, RRID: AB_2167385), goat anti-Brn1 (Novus Biologicals,
1:400, RRID: AB_10012062), mouse anti-NPY-R (E-4) (Santa Cruz Biotechnology, 1:1000,
RRID: AB_2721049), rabbit anti-FoxP2 (Abcam, 1:8000, RRID: AB_2107107), rabbit anti-
Tbr1 (Abcam, 1:1000, RRID: AB_2200219), mouse anti-Dcp1 (Novus Biologicals, 1:1000,
RRID: AB_538184), rabbit anti-Cleaved Caspase 3 (Cell Signaling, 1:500, RRID:
AB_2341188), rat anti-GFAP (Invitrogen, 1:300, catlog no. 130300), rabbit anti-
Doublecortin (Cell Signaling, 1:500, catlog no. 4604), goat anti-Sox2 (R&D Systems, 1:250,
catalog no. AF2018), rabbit anti-Ki67 (Abcam, 1:250, catalog no. ab15580), rat anti-BrdU
(AbD Serotec, 1:300, catalog no. OBT0030) The Alexa350, Alexa488, Alexa555, and
Alexa647-conjugated secondary antibodies were obtained from Invitrogen. HRP-conjugated
goat anti-mouse or anti-rabbit secondary antibodies were purchased from Boehringer Mannheim.

3.6 Immunostaining and histological analysis

Immunocytochemistry on cultured cells was performed as previously described (Zahr et al., 2018). Briefly, cells on glass coverslips were fixed for 15 minutes with 4% buffered paraformaldehyde (PFA), followed by 3 washes with PBS and permeabilization with 0.3% Triton X100 diluted in PBS for 3 minutes. Cells were subsequently blocked with 2% bovine serum albumin (BSA) (Jackson ImmunoResearch Laboratories) in PBS and incubated with primary antibodies in PBS overnight at 4°C. Samples were washed 3 times with PBS, and secondary antibodies, diluted in PBS (1:1000), were added for an additional hour at room temperature. Nuclei were counterstained with Hoechst 33258 (Sigma). Coverslips were mounted on glass slides. For immunostaining of embryonic cortical sections, embryonic brains were dissected in ice-cold HBSS, fixed in 4% paraformaldehyde at 4°C overnight, cryopreserved with 30% sucrose overnight, and placed in OCT at -80°C for at least a few hours. Embryonic brains were cryosectioned coronally at 16μm. For immunostaining of postnatal (P8) cortical sections, postnatal brains were dissected in ice-cold HBSS, fixed in 4% paraformaldehyde at 4°C overnight, cryopreserved with 30% sucrose for 48h, and placed in OCT at -80°C for at least a few hours. Embryonic brains were cryosectioned coronally at 16μm. Postnatal (P8) brains were cryosectioned coronally at 18-20μm Sections were blocked at room temperature with 5% BSA and 0.3% Triton X100 in PBS, and incubated with primary antibodies in 1/2 blocking buffer overnight at 4°C. Sections were washed 3 times with PBS and incubated with appropriate secondary antibodies in PBS at room temperature.
for 1 hour. Sections were counterstained with Hoechst 33258 (Sigma) and mounted as described above.

3.7 Protein immunoprecipitation and immunoblotting

Freshly dissected cerebral cortices from E12-13 mouse embryos were lysed with Gentle Lysis Buffer (GLB) containing 25 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM EGTA, 10 mM NaCl, 0.5% Triton X-100 and 10% glycerol supplemented with the Complete Protease Inhibitor Tablets (Roche Applied Science) and 1mM PMSF. Lysates were precleared by incubating with Protein A/G magnetic beads (Millipore) for 30 min at 4°C, followed by incubation with 5μg mouse anti-4E-T antibody (Novus Biologicals), or normal mouse IgG (Millipore, RRID: AB_145840) at 4°C for 2 hours, followed by a 1 hour incubation with protein A/G magnetic beads at 4°C. Immunoprecipitates were washed three times with GLB buffer, boiled in 2x sample buffer with 1mM dithiothreitol (DTT) for 3 minutes, and analyzed with SDS-PAGE as described previously (Amadei et al., 2015).

3.8 RNA immunoprecipitation (RIP) and microarray analysis

E12-13 cortical lysates used for immunoprecipitations were analyzed using the Magna RIP RNA-Binding Protein Immunoprecipitation Kit in RNase-free conditions on ice, following the manufacturer's instructions (Millipore). Briefly, input lysates were precleared with protein A/G beads and incubated with 5μg rabbit anti-Pumilio2 antibody (MBL) or normal rabbit IgG (Millipore, RRID: AB_145841) for 3 hours at 4°C. Total RNA was isolated from the input lysates and from the immunoprecipitations, extracted with phenol/chloroform, and the quality of RNA was checked on a BioAnalyzer (Agilent). RNA samples from three biological replicates each of total embryonic cortical input lysates, IgG control
immunoprecipitates and Pum2 immunoprecipitates were amplified using the GeneChip® Pico Kit (Thermo Fisher) and subjected to microarray analysis using Mouse Gene 2.0 ST Arrays (Thermo Fisher). The raw data obtained from the microarrays was normalized using robust multiarray analysis in the Expression Console (Thermo Fisher) program. After filtering out probesets for non-protein-coding genes, the limma package in R (Ritchie et al., 2015) was used to calculate log fold changes for IgG RIP over the input, and transcripts with log fold change (LFC) > 0.58 (FC > 1.5) were removed. The log fold changes for Pum2 RIP over input were then calculated. The Pum2 targets were defined as transcripts with LFC > 0.58 (FC > 1.5) and adjusted p-values < 0.05. Similarly, the Pum2 background set included transcripts with LFCs < 0 (FC<1) and adjusted p-values < 0.05. The enriched probe sets were analyzed using DAVID (Huang et al., 2009) and the PANTHER Classification System (Mi et al., 2013).

3.9 PCR

cDNA generated from GeneChip Pico Kit (see RIP and microarray analysis) was used for PCR. PCR was done with amplification for 35 cycles with annealing temperature at approximately 60°C for all primers, using Phusion High-Fidelity DNA Polymerase (NEB). For quantitative real-time PCR, 10 µL PCR reaction mixture containing FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals) was prepared according to the manufacturer's instruction, and loaded on to a 96 multiwell plate. The LightCycler 480 thermocycler (Roche Molecular Biochemicals) was used with a protocol involving an initial activation cycle (2 min, 95°C), 45 cycles of denaturation (10 sec, 95°C), annealing (20 sec, 60°C) and elongation (20 sec, 72°C). A single fluorescence reading was acquired at the end of each elongation step. A melting curve analysis cycle was performed after the PCR
amplification. The primers used in RT-qPCR were: Celsr2 forward 5'-CAC GAT GGC CTG AGG GGT T-3' and reverse 5'-CCT TGT GGA GAA AGG TGT CCT-3'; cox6b1 forward 5'-ACT ACC TGG ACT TCC ACC G-3' and reverse 5'-ACC CAT GAC ACG GGA CAG A-3'; 4E-T forward 5'-GAC TGC ATT CAA CAA GCT AGT GA-3' and reverse 5'-GGG GCC AAT AAG TGA CTT TCA AC-3'; foxf2 forward 5'-CGT CCT CTT CTA ACT CCG TCA-3' and reverse 3'-ATG TAC GAG TAA GGA GGC TTC T-3'; foxq1 forward 5'-AAA TTG GAG GTG TTC GTC CCA-3' and reverse 5'-TCC CCG TCT GAG CCT AAG G-3'; mmd2 forward 5'-AGT ATG AAC ACG CA G CAA ACT-3' and reverse 5'-TCC CAG TCG TCA TCG GAC A-3'; neurog1 forward 5'-CCA GCG ACA CTG AGT CCT G-3' and reverse 5'-CGG GCC ATA GGT GAA GTC TT-3'; neurog2 forward 5'-AAC TCC ACG TCC CCA TAC AG-3' and reverse 5'-GAG GCG CAT AAG GAT GCT TC-3'; neurod1 forward 5'-ATG ACC AAA TCA TAC AGC GAG AG-3' and reverse 5'-TCC CCG TCT GAG CCT TGC TCC TCG T-3'; brn1 forward 5'-AGCAGTTCGCTAAGCAGTTCA-3' and reverse 5'-CGA AGC GG C AGA TAG TGG TC-3'; brn4 forward 5'-CTG CCT CGA ATC CCT ACA GC-3' and reverse 5'-CTG CAA GTA GTC AC TTG GAG AA-3'; prox1 forward 5'-AGA AGC GCG TAG TGG TC-3'; ptpru forward 5'-GCT CAG TAT GAC GAC TTC CAA TG-3' and reverse 5'-GCT CAG TAT GAC GAC TTC CAA TG-3' and reverse 5'-TTG ACC AAG TAG GCA CCA-3'; rabgef1 forward 5'-ATG AGC CTG AAG TCC GAA C-3' and reverse 5'-GCC TTG TGG TAC TCC TCC CT-3'; sepp1 forward 5'-AGC TCT GGC TTG GCC TAC AAA GCC-3' and reverse 5'-CAG TGG TAC TCC TCC TCC CT-3'; Tle3 forward 5'-GAG ACT GAA CAC AAT CCT AGC C-3' and reverse 5'-GGA GTC CAC GTA CCC CGA T-3'; Tle4 forward 5'-CTG GAC AGG TGG TTT GGA CAA-3' and reverse 5'-GAG
3.10 Pum2 motif prediction

For motif prediction, the top 10 n-mers from the position frequency matrix (PFM) inferred by RNAcompete for PUM were generated (Ray et al., 2009) and used to count the number of occurrences of motifs in 3'UTRs of mRNAs from 4ET-RIP target and background sets. To determine how well these motif counts can distinguish between the mRNAs in the target and background sets, we used the area under the ROC curve (AUROC) metric that measures the expected proportion of positives ranked before a randomly drawn negative example. To this end, the mRNAs in the 4ET-RIP target and background sets were labeled with 1 and 0, respectively. The counts for motif occurrences were used as a prediction score. Pum1/2 had an AU-ROC of ~0.79.

Ortholog genes between mouse and human were retrieved from Ensembl through BioMart (http://www.ensembl.org/biomart on November 1, 2016).

3.11 Fluorescence in situ hybridization

The single molecule FISH was performed with probes targeting Brn1 (NM_008900), Tle4 (NM_011600), Tle3 (NM_001083927.1), Diap3 (NM_019670.1), Glo1 (NM_025374.3), Vcam1 (NM_011693.3), Aldoc (NM_009657.3), Ctip2 (NM_021399.2), and EIF4ENIF1/4E-T (NM_023743.2) using the RNAscope kit (Advanced Cell Diagnostics), according to the manufacturer's instructions. Briefly, freshly dissected embryonic brains were fixed overnight with RNAse-free 4% PFA, cryopreserved overnight with RNAse-free 30% sucrose, and
placed in OCT at -80°C overnight. Embryonic brains were cryosectioned coronally at 16μm. Postnatal (P8) brains were cryosectioned coronally at 18-20μm. Sections were washed with ethanol, followed by tissue pretreatment, probe hybridization, and signal amplification. Alternatively, cortical precursor cultures from E12-13 cortices were maintained for 3 days before fixation, ethanol wash, probe hybridization, and signal amplification. In both cases, positive staining was identified as punctate dots present in the nucleus and/or cytoplasm. For simultaneous immunodetection of a particular protein after FISH, sections or cultures were blocked and incubated with the relevant primary antibody overnight at 4°C, followed by 1 hour incubation with the appropriate Alexa-conjugated secondary antibody at room temperature before DAPI staining. Z-stacks of confocal images were taken with optical slice thickness of 0.1 μm. The VZ/SVZ region of 40X confocal images were divided into 5 bins of identical area; the total number of mRNA granules in each bin (~100-200 mRNA granules/bin and ~500-1000 mRNA granules/section) were used for quantification of colocalization. Bright and clear mRNA granules that overlapped with immunostained 4E-T or Pum2 were counted using Volocity software (Perkin Elmer). About 160 Z-stacked images encompassing each bin were used for this analysis.

3.12 Proximity Ligation Assay

PLA was performed as described previously (Amadei et al., 2015) with a DuoLink in situ Red Starter Kit Mouse/Rabbit (Sigma) according to the manufacturer's instructions. Briefly, coverslips were incubated with the appropriate primary antibodies, followed by incubation with the secondary antibodies provided in the kit for 1 hour, followed by ligation reaction for 30 minutes, and signal amplification reaction for 1 hour and 40 minutes. All incubation steps were performed at 37°C in a humidified chamber. Following signal amplification and wash
steps, the coverslips were mounted with the DAPI-containing mounting medium provided in
the kit.

3.13 Single-cell RNA sequencing (scRNAseq)

scRNAseq data collected from the embryonic cortex, using the Drop-seq method, is
described in Yuzwa et al. (2017; GEO: GSE107122) and was analyzed using the same
computational pipeline. scRNAseq data from embryonic ages E13.5, E15.5, and E17.5 was
visualized by t-SNE projections with the overlaid expression of individual genes using a
range of colors from yellow (not detected) to blue/purple (highest expression) using the
FeaturePlot function as implemented in Seurat package in R. To determine the proportion of
cells that express a given specifier gene, the which function in R was used to determine the
number of cells within a cluster or group of clusters with expression values greater than 0. To
determine the proportion of RPs that express a given number of specifier genes, a subset of
the gene expression matrix containing the expression levels of 17 manually curated specifier
genes in only the cells of the RP clusters was used. The number of expressed specifier genes
in each RP was determined using the colSums function in R. Histograms were plotted using
GraphPad Prism 6 software. To determine the average expression level of specifier genes
Brn1 and Tle4 in RPs compared to neurons, a subset of the expression matrix containing only
the RPs and neurons that expressed the aforementioned specifier genes was used and the
average expression level for each gene was determined using the rowMeans function in R.

3.14 Microscopy and quantification

Analysis of cell culture and brain sections were performed as previously described (Wang et
al., 2010). Briefly, cells grown on glass coverslips were analyzed with a Zeiss Axioplan2
microscope. For quantification, 100-300 EGFP-positive transfected cells per condition were counted and results from at least three independent experiments were analyzed. For the analysis of embryonic brains with *in utero* electroporation, at least 3 anatomically matched sections per brain from at least 3 embryos of 2 to 3 independent mothers for each condition were imaged with a 20X objective on an Olympus IX81 fluorescence microscope equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera and Okogawa CSU X1 spinning disk confocal scan head. Images were processed by using Volocity software (Perkin Elmer) and Adobe Photoshop CS6. Pax6, Tbr2 and Hoechst staining were used to define the ventricular zone (VZ), subventricular zone (SVZ) and cortical plate (CP). For the analysis of postnatal brains, 5-6 V-SVZ sections per brain from 3-5 pups of 3 independent mothers for each condition (NestinCreERT2+/−; 4E-Tfl/fl and NestinCreERT2−/−; 4E-Tfl/fl) were imaged with a 20X objective on an Olympus IX81 fluorescence microscope equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera and Okogawa CSU X1 spinning disk confocal scan head. Costes' test for colocalization was performed using ImageJ's "Just Another Colocalization Plugin" (JACoP) (Costes et al, 2004; Bolte et al, 2006). In brief, the test creates randomized images by scrambling pixels of the green channel. This process is repeated 200 times, and the Pearson correlation coefficient (r) is calculated every time between the scrambled image of the green channel and the original unscrambled red channel image. The measured correlation coefficient of the original unscrambled image is subsequently compared to the distribution of correlation coefficients of the randomized images and a probability (P-value) is calculated. P>95%/0.95 suggests significant true colocalization (Costes et al, 2004).
3.15 Statistics

Sample sizes (n) indicated in figure legends 8A, 9B, 18A, 18B, 19A, 19B correspond to the number of independent experiments analyzed (n=3). Sample sizes (n) indicated in figure legends 7D, 7E, 8H, 8I, 10, 11, 12, 13, 17, 19C, 19D correspond to the number of embryos from at least two independent mothers analyzed (n=3-4). All data were expressed as the mean plus or minus the standard error of the mean (SEM), unless otherwise indicated. With the exception of the microarray data, statistical analyses were performed with a two-tailed Student's t-test or, where relevant, ANOVA with Dunnett's or Tukey's post-hoc tests, using GraphPad Prism 6 software. For gene ontology analysis, p-values were determined by DAVID. For the RIP-microarray analysis, the p-values were adjusted with the Benjamini-Hochberg method in R. For the Pum1/2 motif analysis, p-values were determined by the Wilcoxon rank sum test. p-value <0.05 was considered significant. In all figures, asterisks denote statistical significance *p<0.05, **p<0.01, ***p<0.001.

3.16 Data and Software Availability

The Pum2 RNA-immunoprecipitation expression data have been deposited in the GEO database under ID code GEO: GSE108404.
Chapter 4. A Translational Repression Complex in Developing Mammalian Neural Stem Cells that Regulates Neuronal Specification

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This chapter is a modified version of the published manuscript “A translational repression complex in developing mammalian neural stem cells that regulates neuronal specification” (Neuron 2018, 97(3):520-537).

Author Contributions

SKZ conceptualized, designed, performed, and analyzed most of the experiments and co-wrote the paper. GY conceptualized, performed, and analyzed the RIP/microarray experiments and contributed to the qRT-PCR, FISH, culture, and co-immunoprecipitation experiments. HK analyzed and validated the RIP experiments. SAY and MJB performed and analyzed the scRNAseq experiments and MJB participated in FISH analysis. AV performed and analyzed the qPCRs. DRK conceptualized experiments and co-wrote the paper. FDM conceptualized and designed experiments, analyzed data, and co-wrote the paper.
4.1 Abstract

The mechanisms instructing genesis of neuronal subtypes from mammalian neural precursors are not well-understood. To address this issue, we have characterized the transcriptional landscape of radial glial precursors (RPs) in the embryonic murine cortex. We show that individual RPs express mRNA but not protein for transcriptional specifiers of both deep and superficial layer cortical neurons. Some of these mRNAs, including the superficial versus deep layer neuron transcriptional regulators Brn1 and Tle4, are translationally repressed by their association with the RNA-binding protein Pumilio2 (Pum2) and the 4E-T protein. When these repressive complexes are disrupted in RPs mid-neurogenesis by knocking down 4E-T or Pum2, this causes aberrant co-expression of deep layer neuron specification proteins in newborn superficial neurons. Thus, cortical RPs are transcriptionally primed to generate diverse types of neurons, and a 4E-T-Pum2 complex represses translation of some of these neuronal identity mRNAs to ensure appropriate temporal specification of daughter neurons.

4.2 Introduction

Appropriate circuit assembly in the mammalian cerebral cortex requires the genesis of diverse excitatory neurons that differ in their morphology, connectivity, and function. These different neurons are all made by radial glial precursors (RPs) that generate neurons either directly or indirectly via transit-amplifying intermediate progenitor cells (IPs). The newborn neurons then migrate basally to form the nascent cortical layers, with the earliest-born neurons populating the deepest layers, and later-born neurons progressively populating
more superficial layers. Subsequent to this neurogenic period, which occurs from E11 to E17 in the mouse, the same pool of RPs generates glial cells.

What determines this timed neuronal genesis, particularly in light of recent work showing that individual cortical RPs are multipotent and sequentially generate diverse cortical neurons (Guo et al., 2013; Gao et al., 2014; Eckler et al., 2015; Shen et al., 2006)? One attractive molecular explanation posits transcriptional induction of regulatory proteins that specify neuron subtypes as neurons are generated (Greig et al., 2013; Kwan et al., 2012). However, this model is complicated by the finding that RPs themselves express some neuronal specifiers at the mRNA but not protein levels (Arlotta et al., 2005; Guo et al., 2013; Eckler et al., 2015), indicating that post-transcriptional regulation might also be important. In this regard, we recently identified a translational repression complex involving the 4E-T protein and showed that it determines the timing and extent of cortical neurogenesis by regulating translation of proneurogenic bHLH proteins (Yang et al., 2014). These findings suggest a second, not mutually exclusive, model where cortical RPs are transcriptionally primed to make diverse neuronal subtypes, and where selective repression determines which specifiers are translated and thus, which types of neurons are generated.

Here, we have tested this model and provide evidence that during embryonic neurogenesis, cortical RPs co-express mRNAs encoding specifiers for diverse cortical neuron subtypes, and that a complex involving 4E-T and the RNA-binding protein Pumilio2 selectively represses translation of some of these mRNAs to ensure the appropriate specification of daughter neurons.
4.3 Results

Single-cell RNA sequencing demonstrates that embryonic RPs co-express mRNAs encoding specification factors for different types of cortical neurons

To identify neuronal specification genes expressed by embryonic precursors, we analyzed recently-published single-cell RNA sequencing (scRNAseq) data from the murine cortex obtained at embryonic day 13.5 (E13.5), when both deep and superficial layer neurons are generated, at E15.5 when only superficial layer neurons are made, and at E17.5 when neurogenesis is over (Yuzwa et al., 2017; GEO: GSE107122). This study used Drop-seq to transcriptionally profile 2000-5000 total cortical cells at each age and to define RPs, IPs and neurons (Figure 14A). We focused on the RP clusters in these datasets, which included 233, 273 and 77 cells at E13.5, E15.5 and E17.5, respectively (Fig. 14A).

We first analyzed the E13.5 and E15.5 RP transcriptomes for expression of 26 genes encoding proteins that specify and/or are associated with cortical neurons in different layers (termed specification genes). These included 13 genes for superficial layer neurons (Pou3f3/Brn1, Pou3f2/Brn2, Lhx2, Cux1, Tle3, Tle1, Mef2c, Bhlhe22/Bhlhb5, Cux2, Pou3f1/Oct6, Kitz, Unc5d, and Satb2) and 13 for deep layer neurons (Tle4, Fezf2, Ctip2, Otx1, Sox5, Lix1, Lmo4, Diap3, Lxn, Foxp2, Tbr1, Ldb2 and Pcp4). All of these mRNAs were detectably-expressed in E13.5 neurons (see Fig. 14B for examples; Yuzwa et al., 2017). Six superficial layer (Pou3f3/Brn1, Pou3f2/Brn2, Lhx2, Cux1, Tle3 and Tle1), and nine deep layer neuron mRNAs (Tle4, Fezf2, Ctip2, Otx1, Sox5, Lix1, Lmo4, Diap3 and FoxP2) were detectably expressed in 6% to 55% of E13.5 and E15.5 RPs (Fig. 6A,B). Visualizations using t-distributed stochastic neighbour embedding (t-SNE) indicated that amongst the most
widely-detected were the superficial layer specifiers Pou3f3/Brn1 (51 to 55%) and Cux1 (33 to 34%), and the deep layer specifiers Fezf2 (21 to 29%) and Ctip2 (16 to 21%) (Fig. 6A,B). The remaining 11 genes were detectably expressed in ≤5% of E13.5 RPs [Bhlhe22 (1%), Cux2 (0.5%), Pou3f1/Oct6 (3%), Kitl (3%), Unc5d (2%), Satb2 (3%), Mef2c (2%), Lxn (5%), Ldb2 (2%), Pcp4 (4%), Tbr1 (2%)] and were not further analyzed except for Bhlhe22 and Tbr1, which were included as examples of neuron-enriched specification genes (Fig. 14B).

The t-SNE visualizations also showed that many RPs co-expressed deep and superficial layer neuron specification mRNAs (Fig. 6C). We quantified this by determining the proportion of E13.5 and E15.5 RPs that co-expressed the 15 superficial versus deep layer genes expressed in more than 5% of the RPs plus Bhlhe22 and Tbr1 (that is, the genes in Fig. 6B). At both ages, ≥95% of cells in the RP clusters expressed at least one specification mRNA, and 72-73% co-expressed both superficial and deep layer mRNAs. A distribution analysis (Fig. 6E) showed that >50% of RPs at E13.5 and E15.5 expressed 3 or more specification genes (see Fig. 6C for examples), and that about 10% expressed 6 to 10. Thus, from E13.5 to 15.5 most RPs are transcriptionally primed to make diverse cortical neurons. A similar analysis at E17.5, when neurogenesis is over, showed that all of the specification genes were still detectably expressed in at least some RPs (Fig. 6A,B), and that many RPs still co-expressed superficial and deep layer neuron specification genes (Fig. 6D). However, individual E17.5 RPs did not express as many specification genes as at the earlier time points, and very few expressed 6 or more (Fig. 6E,F).
**Figure A**

 Heatmaps showing the expression of Pou3f3/Brn1 and Cux1 in E13.5, E15.5, and E17.5.

**Figure B**

<table>
<thead>
<tr>
<th>Gene</th>
<th>% E13.5 RPs</th>
<th>% E15.5 RPs</th>
<th>% E17.5 RPs</th>
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<tr>
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</tr>
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**Figure C**

Heatmaps showing the expression of Hes5, Brn1, Brn2, Tle3, Tle1, Pax6, Tle4, Ctip2, Fezf2, and Diap3 in E15.5.

**Figure D**

<table>
<thead>
<tr>
<th>Age</th>
<th>% RPs expressing both DL and SL specifiers</th>
<th>% RPs expressing only SL specifiers</th>
<th>% RPs expressing only DL specifiers</th>
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<td>E17.5</td>
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</table>

**Figure E**

Bar graph showing the percentage of RPs expressing each specifier in E13.5, E15.5, and E17.5.

**Figure F**

Bar graph showing the average number of specifiers per RP in E13.5, E15.5, and E17.5.
Figure 6. Individual embryonic RPs detectably co-express specification mRNAs as determined by scRNAseq. (Also see Fig. 14). Analysis of scRNAseq data from the E13.5, E15.5 and E17.5 RP clusters in Yuzwa et al. (2017; GEO: GSE 107122) (see Fig. 14). (A) t-SNE visualization of E13.5, E15.5 and E17.5 scRNAseq data overlaid with expression of the superficial layer (SL) specifiers Pou3f3/Brn1 and Cux1 and the deep layer (DL) specifiers Ctip2 and Fezf2. Cells are colour coded according to expression level ranging from not detected (yellow) to the highest detected levels (blue), according to the adjacent color key. Boxed regions in the E15.5 panels are shown at higher resolution in panel (C). (B) Table showing the percentages of E13.5, E15.5, and E17.5 RPs expressing superficial and deep layer neuron specifiers. (C) Higher resolution t-SNE visualizations of E15.5 RPs from the boxed regions in (A) showing overlaid expression of Hes5, Brn1, Brn2, Tle3, Tle1, Pax6, Tle4, Ctip2, Fezf2 and Diap3 mRNAs. Gene expression levels are colour coded as in (A). Arrowheads denote individual RPs co-expressing superficial and deep layer specifier mRNAs. (D) Table showing the percentage of E13.5, E15.5, and E17.5 RPs expressing both superficial and deep layer neuron specifiers, only superficial layer specifiers, or only deep layer specifiers. (E) Histograms showing the number of specification mRNAs, of the 17 listed in (B), that were detected in individual RPs at E13.5, E15.5 and E17.5, expressed as a percentage of the total RPs at the same age. (F) Average number of specification mRNAs detected in individual RPs at E13.5, E15.5 and E17.5. **p<0.01, ***p<0.001, ns=p>0.05 by one-way ANOVA with Tukey's multiple comparisons test; n=233, 273, and 77 RPs for E13.5, E15.5 and E17.5 respectively. Error bars denote SEM.

Deep and superficial layer neuron specification mRNAs are co-expressed in RPs throughout neurogenesis

We further characterized the co-expression of neuronal specification genes in RPs by performing single molecule fluorescence in situ hybridization (FISH). We focused initially on Brn1 mRNA, since it had widespread expression in RPs, as indicated by the scRNA seq data (Fig. 6A,B; Fig. 14B), and because it is important for superficial layer neurogenesis (Sugitani et al., 2002; Dominguez et al., 2013). We analyzed the cortex at E12, before
superficial layer neurons are generated. Immunostaining with an antibody that recognizes both Brn1 and Brn2 combined with FISH (Fig. 7A; Fig. 15A) showed that Brn1/Brn2 protein was undetectable in the medial cortex, as previously published (Dominguez et al., 2013), but that Brn1 mRNA was expressed in most Pax6-positive RPs. We asked if these Brn1 mRNA-positive RPs co-expressed other neuronal specification mRNAs, analyzing Tle4 (layer V/VI), Tle3 (layer II/III), and Diap3 (predominantly layer V) mRNAs. Multi-label FISH at E12 and E13 showed that many RPs co-expressed these mRNAs and that about 70% of cells in the E13 precursor-containing ventricular and subventricular zones (VZ/SVZ) co-expressed Brn1, Tle4 and Diap3 mRNAs (Fig. 7B-D).

A similar analysis at E15 and E17 (Fig. 7D-F) showed that the proportion of VZ/SVZ cells co-expressing Brn1, Tle4 and Diap3 mRNAs decreased from E13 to E17, but that approximately 30% of E17 RPs still co-expressed all 3 mRNAs. Coincidentally, there was an increase in VZ/SVZ cells that expressed only Brn1 mRNA (Fig. 7E). Triple-label FISH with negative control probes demonstrated the specificity of these analyses (Fig. 15B,C).

We confirmed the co-expression of neuronal specification mRNAs in RPs by analyzing cultured E12.5 cortical precursors that generate neurons in vitro. Triple-label FISH combined with immunostaining (Fig. 7G,H) showed that many βIII-tubulin-negative precursors co-expressed Brn1, Tle4 and Diap3 mRNAs, although some were also positive only for Brn1 mRNA. Intriguingly, about 30% of newborn βIII-tubulin-positive neurons also co-expressed Brn1, Tle4 and Diap3 mRNAs (Fig. 7G, top panel), although many expressed only Brn1 mRNA and some only Tle4 mRNA (Fig. 7H).
We asked whether this neuronal co-expression was also seen in vivo, examining the cortex at P3 when neurogenesis is complete. Immunostaining (Fig. 7I) confirmed that, as previously published (Yao et al., 1998; Dominguez et al., 2013), Brn1/2 and Tle4 proteins were detectably expressed in mutually-exclusive superficial and deep layer neurons, respectively. In contrast, FISH showed that in superficial layers II-IV, where there were no Tle4-positive cells, some Brn1/2 protein-positive cells expressed both Brn1 and Tle4 mRNAs (Fig. 7J). Indeed, triple-label FISH showed that some neurons in the most superficial layers co-expressed Brn1, Tle4 and Diap3 mRNAs (Fig. 7K). Conversely, in layer VI, where there were no Brn1/2 protein-positive cells, some Tle4-protein positive cells co-expressed both Tle4 and Brn1 mRNAs (Fig. 7L). Thus, newborn cortical neurons appropriately express laminar specification proteins, but at the transcriptional level some of them are more promiscuous.
Figure 7. Developing cortical RPs and newborn neurons co-express mRNAs associated with superficial and deep layer cortical neurons. (Also see Fig. 15). (A) Representative high magnification confocal z-stack images of the E12 cortical VZ showing FISH for Brn1 mRNA (green, left and center left), and immunostaining for Pax6 (turquoise, right) and Brn1/2 protein (red, center right; not detectable at this age). The merged image (left) shows Brn1/2 protein, Brn1 mRNA and Hoechst 33258 counterstain (dark blue) but not Pax6. The hatched white lines denote the apical cortical border with the lateral ventricle (LV). A corresponding low magnification image of the same section is shown in Fig. 15A. (B, C) Representative confocal z-stack images of multi-label FISH for Brn1 (green), Tle3 (red) and Diap3 (blue) (B) or Brn1 (red) and Tle4 (green) (C) mRNAs in E12 coronal cortical sections, showing the VZ and the apical border (hatched white line). The boxed areas are also shown at higher magnification to the right and single cell nuclei are highlighted (outlined in white; Hoechst counterstain is light blue). (D, E) Quantification of images as in (B, C and F) for the proportion of VZ/SVZ cells expressing Brn1, Diap3, and Tle4 mRNAs (D) or Brn1 mRNA only (E) at E13, E15, and E17. *p<0.05, **p<0.01, ***p<0.001; n = 3 embryos per time point, 100 cells per embryo. LV = lateral ventricle. (F) Representative confocal z-stack image of multi-label FISH for Brn1 (red), Tle4 (blue) and Diap3 (green) mRNAs in an E17 coronal cortical section, showing the VZ/SVZ and the apical border (hatched white line). The boxed area is also shown at higher magnification on the bottom and a single cell nucleus is highlighted (outlined in white; Hoechst counterstain is light blue). (G, H) Representative z-stack images of cortical cultures immunostained for βIII-tubulin (purple) and analyzed by FISH for Brn1 (red), Tle4 (blue), and Diap3 (green) mRNAs. The arrow in the top image in (G) denotes a βIII-tubulin-positive cell co-expressing all three mRNAs while the arrow in the lower image denotes a βIII-tubulin-negative cell expressing only Brn1 mRNA. (I) Representative images of a P3 cortical section immunostained for Brn1/2 (green) and Tle4 (red) and counterstained with Hoechst 33258 (blue in the left merged image). Arrows and arrowheads denote Tle4-positive and Brn1/2-positive neurons respectively. Hatched lines delineate boundaries between layers II–IV, V and VI. (J) High-magnification confocal image showing FISH for Brn1 (red) and Tle4 (blue) mRNAs and immunostaining for Brn1/2 (green) in the superficial layers (II-IV) of the P3 cortex. Arrows indicate neurons co-expressing Brn1 and Tle4 mRNAs and one of these (circled) is shown at
higher magnification to the right with the color channels pulled apart. **(K)** Representative confocal z-stack images showing FISH for *Brn1* (red), *Tle4* (blue) and *Diap3* (green) mRNAs in the superficial layers (II/III) of the P3 cortex. Boxed cells are shown at higher magnification on the right, with the color channels pulled apart. White ovals denote cell boundaries defined by Hoechst nuclear staining (light blue/grey in left merged image). **(L)** High-magnification confocal image showing FISH for *Brn1* (green) and *Tle4* (blue) mRNAs and immunostaining for Tle4 (red) in layer VI of the P3 cortex. Arrows denote neurons co-expressing *Brn1* and *Tle4* mRNAs and one of these (circled) is shown at higher magnification to the right, with the color channels shown separately. Scale bars, 10µm in (A) and (K, low magnification), 5µm in (B, C,F, G, H, J, L) and (K, high magnification), 30 µm in (I). Error bars denote SEM.

**Identification of a Pum2-4E-T translational repression complex in embryonic RPs**

These data suggest that post-transcriptional regulation is important for neuronal specification. Since we showed that the translational repressor protein 4E-T regulates the extent and timing of cortical neurogenesis (Yang et al., 2014), we asked if it might also be in a complex with neuronal specification mRNAs. Analysis of our previously-published 4E-T RNA immunoprecipitation (RIP) data from the E12.5 cortex showed that *Brn1*, *Tle3*, *Tle4*, *Mef2c*, *Bhlhe22* and *Diap3* mRNAs were all significantly associated with 4E-T (adjusted p-values - *Brn1*: 1.75x10⁻³, *Tle3*:8.53x10⁻⁴, *Tle4*:2.81x10⁻³, *Mef2c*: 1.55x10⁻³, *Bhlhe22*: 2.72x10⁻⁶, *Diap3*: 6.13x10⁻²). We confirmed the association of 4E-T with *Brn1*, *Tle3* and *Tle4* mRNAs in the RIPs by performing qPCR analysis (Fig. 8A).

Since 4E-T does not directly bind RNA, we asked whether the 3' UTRs of cortical mRNAs associated with 4E-T were enriched in RNA binding-protein consensus elements, as predicted by RNAcompete (Ray et al., 2009). This analysis showed that Pumilio1/2
(Pum1/2) consensus motifs significantly distinguished 4E-T target mRNAs from background mRNAs (AU-ROC = 0.79; see experimental methods). Of particular relevance, Brn1, Tle3, Tle4, Bhlhe22, Diap3, and Mef2c mRNAs all had computationally-predicted Pum1/2 consensus sites (Table 1).

Since Pumilio proteins are known translational repressors (Wickens et al., 2002; Miller and Olivas, 2011; Quenault et al., 2011), and Pum2 is expressed in embryonic cortical RPs (Vessey et al., 2012), we asked if Pum2 and 4E-T were associated in the embryonic cortex. Four lines of evidence indicated that they were. First, western blots showed that Pum2 was present in anti-4E-T immunoprecipitated complexes from the E12/13 cortex (Fig. 8B). Second, immunostaining of cultured E12/13 cortical precursors showed that Pum2 and 4E-T were both present in cytoplasmic granule-like structures, and that about 65% of Pum2-positive puncta were also positive for 4E-T (Fig. 8C). Moreover, as previously seen for 4E-T (Yang et al., 2014), many Pum2-positive puncta were also positive for the P-body protein Dcp1 (Fig. 8D). Third, proximity ligation assays identified foci in cultured cortical precursors where Pum2 was located within 40 nm of 4E-T or Dcp1 (Fig. 8E,F). In contrast, proximity ligation assays for Pum2 and the NPY1 receptor or Pax6 showed only a few background dots (Fig. 16).

Fourth, we asked whether Pum2 and 4E-T were associated in vivo. Immunostaining of E13 cortical sections (Fig. 8G) showed that Pum2-positive puncta were present throughout the cortex, and were significantly enriched in the apical-most region of the VZ (Fig. 8G,H). In this same apical region, about 50% of Pum2-positive puncta were also positive for 4E-T (Fig. 8G, I). To ensure this colocalization was specific, we randomized the images (Costes et
al., 2004). For the original 4E-T/Pum2 data, Pearson's coefficient was $r = 0.541$ and for the randomized data it was $r = 0.0 \pm 0.009$ (p = 100% that colocalization was not random).
Figure 8: Pum2 and 4E-T are closely associated in embryonic RPs. (Also see Fig. 16). (A) qPCR validation for Tle3, Tle4, and Brn1 mRNAs in 3 independent 4E-T and control IgG immunoprecipitates and their initial inputs. Shown is fold enrichment of each mRNA relative to input. **p<0.01 (pairwise comparison to IgG RIP). (B) Western blots of E12.5 cortical lysates (input) immunoprecipitated (IP) with control IgG (IgG) or anti-4E-T (4E-T), probed for 4E-T or Pum2. Arrowheads denote target proteins. (C, D) Representative images of E12 precursors cultured 3 days, immunostained for Pum2 (red) and 4E-T (green, C) or Dcp1 (green, D) and counterstained with Hoechst 33258 (blue). Boxed regions in (C) are shown at higher magnification (left), and also indicate colocalization on the z-axis (XZ and YZ) with hatched lines. In (D), arrows and arrowheads indicate Pum2 foci positive or negative for Dcp1 respectively. (E, F) Representative confocal z-stack images of E12 precursors cultured 3 days and analyzed by PLA with antibodies for Pum2 and 4E-T (E) or Dcp1 (F). Cells are counterstained with Hoechst 33258 (blue) and gold dots are the PLA signal. Boxed regions are expanded on the right and single nuclei are demarcated (hatched lines). (G) Confocal images of an E13 section immunostained for Pum2 (green) and 4E-T (red), counterstained with Hoechst 33258 (blue in merged image). Boxed regions are expanded in the right panels. Arrows and arrowheads denote Pum2 foci that are or are not colocalized with 4E-T. Hatched white lines denote the 4 bins used for quantification. LV = lateral ventricle. (H, I) Quantification of images as in (G) for the percentage of total Pum2 granules in each of the 4 bins (H), or for the percentages of Pum2 granules in each bin that were also positive for 4E-T (I). ***p<0.001 by one-way ANOVA with Dunnett's multiple comparisons test; n=3 embryos. Scale bars, 5µm. Error bars denote SEM.

Pum2 and 4E-T share target mRNAs, including neuronal specification mRNAs

These data predict that some 4E-T target mRNAs would be associated with Pum2.

To test this prediction, we immunoprecipitated Pum2 from the E12 cortex, and analyzed the coimmunoprecipitated mRNAs by microarrays (GEO: GSE108404). As controls, we performed similar immunoprecipitations with non-specific IgG. We analyzed these
microarray datasets (three independent replicates each of the Pum2 and IgG immunoprecipitations), first removing (i) all non-protein-coding genes, and (ii) genes with IgG/input fold change of greater than 1.5. We then defined the Pum2 target set as those remaining mRNAs that were enriched more than 1.5-fold in the Pum2 RIP versus input, with a p-value < 0.05. We also defined a background set, including mRNAs that were not enriched in the Pum2 RIP (fold change of less than 1) with a p-value < 0.05. This analysis defined 1783 probes as Pum2 targets and 2806 as the background set (Table 2). Of the 1783 Pum2 target mRNAs, 282 were also 4E-T target mRNAs that had Pum1/2 consensus motifs in their 3' UTRs (Table 1), including Tle3, Tle4, Neurog1, Neurog2, Ascl1, and Mef2c mRNAs. Brn1 mRNA, which is a 4E-T target that contains consensus Pum1/2 motifs (Table 1), was also significantly associated with Pum2 in the RIP dataset (p = 0.017), but was enriched only 1.3-fold. Other mRNAs defined as shared Pum2 and 4E-T targets that encoded transcriptional regulators were Arid1a, Bcl6, Ets2, E2f3, Gli2, Klf6, Mkl1, Meis1, Nkrf, Phf12, Pou3f4, Prdm16, Sox13, Sox2, Rere, Bhlhe40, Cbx4, Cbx8, Elp3, Epc2, Foxc1, Foxk1, Irf2bp1, Jun, Lin54, Maml1, Med23, Mn1, Myc, Mef2a, Nrip1, Nfya, Pias1, Rnf44, Sal13, Stat3, Six4, Txnip, Sp8, Tfap2a, Usp22, Mafb, Mycn, Zbtb14, Zfp11, Zfp143, Zfp229, Zfp273, Zfp282, Zfp518a, Zfp763, Zfp85, Zic1, Zic3, and Zhx1.

We validated the Pum2 RIP dataset in a number of ways. First, computational analysis defined significantly more Pum1/2 motif occurrences in the 3'UTRs of Pum2 target mRNAs versus a set of background mRNAs not enriched in the Pum2 RIP (Fig. 9A). Second, qPCR of three independent Pum2 RIP experiments confirmed that Tle3, Tle4, Brn1, Neurog1, and Neurog2 mRNAs were all significantly enriched in the Pum2 RIPS (Fig. 9B). Finally, we performed a correlation analysis, comparing relative fold-changes in mRNA
levels as determined by microarrays versus qPCRs. For this comparison, we analyzed the shared Pum2 and 4E-T target mRNAs Tle3, Tle4, Brn1, Neurog1 and Neurog2 mRNAs and 8 other Pum2 target mRNAs (4et, Brn4, Prox1, Celsr2, Foxq1, Ptpu, Rabgef1 and Tspan14). We also included four mRNAs (Foxf2, Sepp1, Cox6b1, Mmd2) that were not enriched in the Pum2 RIPs. There was an excellent correlation (r = 0.918) between the fold-changes obtained using microarrays versus qPCRs (Fig. 9C).

We next used DAVID to perform gene ontology on (i) all Pum2 target mRNAs in the RIP dataset, and (ii) the subset of these that were also 4E-T target mRNAs. Total Pum2 targets were highly enriched for proteins associated with cell adhesion, cell migration, transcription, cell differentiation, cell cycle, and forebrain, neuron and stem cell development (Fig. 9D; Table 3). The Pum2/4E-T shared targets were particularly enriched for proteins associated with transcriptional regulation and nervous system/neuronal development (Fig. 9E; Table 3). We obtained a similar enrichment for transcriptional regulators when the Pum2/4E-T dataset was analyzed by PANTHER. Of 282 shared target mRNAs, 126 encoded proteins assigned to categories by PANTHER and the most enriched group included 37 transcription factors (Table 4).
Cumulative fraction

Number of motifs

Pum2 Motif Counts (RNAcompete PWM)

<table>
<thead>
<tr>
<th>Enrichment Score (-log_{10}[p-value])</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell adhesion</td>
</tr>
<tr>
<td>Cell migration</td>
</tr>
<tr>
<td>Positive regulation of transcription</td>
</tr>
<tr>
<td>Multicellular organism development</td>
</tr>
<tr>
<td>Cell differentiation</td>
</tr>
<tr>
<td>Neuron projection development</td>
</tr>
<tr>
<td>Cell cycle</td>
</tr>
<tr>
<td>Forebrain development</td>
</tr>
<tr>
<td>Negative regulation of cell prolif.</td>
</tr>
<tr>
<td>Stem cell population maintenance</td>
</tr>
</tbody>
</table>

Enrichment Score (-log_{10}[p-value])

| Regulation of transcription       | 50 |
| Nervous system development        | 55 |
| Cell differentiation              | 60 |
| Pattern specification process     | 65 |
| Cell fate commitment              | 70 |
| Neuron migration                  | 75 |
| Negative regulation of cell prolif.| 80 |
| Stem cell population maint.       | 85 |
| Positive regulation of neuron differ. | 90 |
| Regulation of neurogenesis        | 95 |

**Gene expression**

**Neuron development**

**Cell growth**

**Cellular movement**

Pax6/βIII-tubulin

Tbr2

Brn1 mRNA

Tle4 mRNA

4E-T

Pum2

Brn1

Tle4
Figure 9. Pum2 and 4E-T share target mRNAs (A-E) Pum2 was immunoprecipitated from the E12/13 cortex, and associated mRNAs were analyzed by microarray (GEO: GSE108404). As a control, similar immunoprecipitations were performed with non-specific IgG. Three independent samples of each were analyzed. The Pum2 target set was defined as the 1768 mRNAs with enrichment of greater than 1.5-fold (p<0.05) versus the input, while the background set included 2684 mRNAs that were not enriched in the Pum2 RIP (fold change of less than 1; p<0.05) (see Table 2 for lists of these mRNAs). (A) The 3'UTRs of mRNAs from the Pum2 RIP target and background datasets were analyzed for occurrences of the top 10 Pum1/2 n-mer consensus motifs predicted by the RNAcompete position frequency matrix (PFM). Motif occurrences were counted, and the cumulative distribution function (CDF) of motif counts are displayed separately for the two groups of mRNAs. The CDF calculates the cumulative probability for a given motif count, and this is significantly larger in the target versus background datasets (p=6.80 x 10^{-101}, Wilcoxon rank sum test). (B) qPCR validation of Brn1, Tle3, Tle4, Neurog1, and Neurog2 mRNAs in 3 independent Pum2 and control IgG immunoprecipitates and their initial inputs. Shown is fold enrichment of each mRNA relative to input. *p<0.05, **p<0.01, ***p<0.001 (pairwise comparison to IgG RIP). (C) Correlation of average fold change (immunoprecipitates/input; n=3) in the Pum2 RIP microarray versus qPCR analyses for the shared Pum2/4E-T target mRNAs in (B), 8 other Pum2 target mRNAs (4et, Prox1, Brn4, Celsr2, Foxq1, Ptpru, Rabgef1, and Tspan14) and 4 mRNAs that were not enriched in the Pum2 RIPs (Foxf2, Sepp1, Cox6b1, Mmd2). Each point represents one of these mRNAs. Pearson’s correlation coefficient = 0.918, p=0.00001. (D, E) Gene ontology and pathway analysis for all the Pum2 target mRNAs in the E12/13 cortex (Tables 2 and 3) (D) and for the 282 shared Pum2 and 4E-T target mRNAs (Tables 3 and 4) (E). Shown are the top ontology terms ranked by their enrichment score (-log10 [p-value], x axis; determined by a modified Fisher's exact test), and the number of genes in each category. In (E) the terms were classified into four color-coded groups based upon their biological functions. Some genes are annotated in several categories. (F) Confocal images of E12 medial cortex sections immunostained for 4E-T (green), Pum2 (green), Brn1 (green; not detectable at this age), and Tle4 (red), or analyzed by FISH for Brn1 or Tle4 mRNA (both white dots). Also shown are immunostaining for Pax6 (red), Tbr2 (red) and βIII-tubulin (blue) to define the VZ, SVZ and
CP, respectively. The top left panel shows the 5 VZ/SVZ bins used for quantification. Scale bars, 30µm. Error bars denote SEM.

**Pum2 and 4E-T are associated with Brn1 and Tle4 mRNAs in apical RPs during cortical neurogenesis**

The shared Pum2 and 4E-T transcription factor targets included *Brn1* and *Tle4* mRNAs. Since these mRNAs are co-expressed during neurogenesis, we asked if they were associated with Pum2 and/or 4E-T in RPs. Initially, we used immunostaining and FISH to define their expression patterns in the E12 cortex during deep layer neurogenesis. At this age, 4E-T and Pum2 proteins were detectable throughout the cortex (Fig. 9F). Brn1 protein was not detectable but *Brn1* mRNA was present in all cortical layers (Fig. 9F), in agreement with the E13.5 scRNAseq data (Fig. 14B), which also showed that average *Brn1* mRNA levels in *Brn1*-positive RPs and neurons were similar (1.34 and 1.38 arbitrary units, respectively). In contrast to Brn1, at E12 Tle4 protein was present in cells from the VZ/SVZ to the CP (Fig. 9F), consistent with ongoing deep layer neurogenesis. *Tle4* mRNA was also distributed across the E12 cortex (Fig. 9F), in agreement with the E13.5 scRNAseq showing similar expression levels in *Tle4*-positive RPs and neurons (0.97 and 1.33 arbitrary units, respectively). As controls for specificity of the FISH, we showed that two RP markers, *Vcam1* and *Aldoc* mRNAs, were found only in the VZ/SVZ of the E13 cortex (Fig. 17A).

We next performed colocalization studies, combining immunostaining and FISH. Quantification of *Brn1* mRNA in the CP and 5 equal-sized bins spanning the VZ/SVZ confirmed that it was equally distributed across the cortex (Fig. 9F; 10A, C). Approximately half of *Brn1* mRNA foci were colocalized with 4E-T in the apical-most precursors (Bin 1) and in the CP, with significantly less colocalization in other regions (Fig. 10A,D). A similar
high level of colocalization between Pum2 and Brn1 mRNA was found in the apical-most RPs (Fig. 10B,E), with 30-40% colocalization in other cortical compartments. A similar analysis for Tle4 mRNA confirmed it was also equally distributed across the cortex (Fig. 9F; 5F,H). However, only about 20% of Tle4 mRNA was associated with 4E-T in the apical-most RPs (Bin 1), with less association elsewhere (Fig. 10F,I). Colocalization with Pum2 was also lower, with approximately 29% of Tle4 mRNA foci associated with Pum2 in the VZ, and less elsewhere (Fig. 10G,J).

Two controls demonstrated the specificity of these analyses. First, we randomized the data (Costes et al., 2004). For the original versus randomized 4E-T/Brn1 mRNA data, Pearson's coefficients were \( r = 0.271 \) and \( r = 0.0 \pm 0.033 \), respectively (\( p = 100\% \)). For the original versus randomized 4E-T/Tle4 mRNA data, \( r = 0.198 \) and \( r = 0.0 \pm 0.057 \) (\( p = 100\% \)). For the original versus randomized Pum2/Brn1 mRNA data, \( r = 0.465 \) and \( r = 0.0 \pm 0.038 \) (\( p = 100\% \)). For the original versus randomized Pum2/Tle4 mRNA data, \( r = 0.357 \) and \( r = 0.0 \pm 0.008 \) (\( p = 100\% \)). Second, we performed a colocalization analysis for Glo1 mRNA, which is not a target of Pum2 or 4E-T. Less than 19% of Glo1 mRNA foci were colocalized with Pum2 in the E12 VZ/SVZ (Fig. 17B,C), and our previously-published data showed a similar low level of colocalization (10-13%) of Glo1 mRNA with 4E-T in the VZ/SVZ (Yang et al., 2014). Thus, both Brn1 and Tle4 mRNAs colocalize with 4E-T and Pum2 in E12 apical RPs, but Brn1 mRNA is more highly colocalized.

These data showed that about 50% of Brn1 mRNA was associated with 4E-T in E12 apical RPs. We asked if this was also true at E16 during superficial layer neurogenesis. As seen at E12, 4E-T and Pum2 proteins and Brn1 mRNA were all expressed throughout the cortex (Fig. 10L; Fig. 17D,E). However, at this age Brn1 protein was also readily detectable
in cells located from the SVZ to the CP (Fig. 17D), as previously published (Dominguez et al., 2013). Quantification showed that Brn1 mRNA was equally distributed across the VZ/SVZ, and that it was colocalized with 4E-T in the apical-most RPs, but that the association with 4E-T was significantly decreased relative to E12 by more than two-fold (Fig. 10K-N). Thus, translation of Brn1 protein during superficial layer neurogenesis is associated with a decreased association between Brn1 mRNA and 4E-T in RPs.
Figure 10. Cortical neuron specification mRNAs are associated with 4E-T and Pum2 in embryonic RPs. (Also see Fig. 17). (A, B) Confocal images showing FISH for Brn1 mRNA (red) and immunostaining for 4E-T (green, A) or Pum2 (green, B) in the E12 VZ. Sections were counterstained with Hoechst 33258 (blue). Arrows denote colocalized foci. The right panels are higher magnification images of the boxed regions that also show colocalization on the z-axis (XZ and YZ; indicated by hatched white lines). (C-E) The E12 VZ/SVZ was divided into five equal bins and the CP was considered as a separate bin (see Fig. 9F). Sections as in (A,B) were then quantified for the percentage of total Brn1 mRNA foci in each bin (C) or for the percentages of Brn1 mRNA foci within a bin that were colocalized with 4E-T (D) or Pum2 (E). ***p<0.001 by one-way ANOVA with Dunnett's multiple comparisons test; n = 3 embryos each. (F, G) Confocal images showing FISH for Tle4 mRNA (red) and immunostaining for 4E-T (green, F) or Pum2 (green, G) in the E12 VZ. Sections were counterstained with Hoechst 33258 (blue). Arrows denote colocalized foci. The right panels are high magnification images of the boxed regions that also show colocalization on the z-axis (XZ and YZ; indicated by hatched white lines). (H-J) The E12 cortex was subdivided as in (C-E) and sections as in (F,G) were then quantified for the percentage of total Tle4 mRNA foci in each bin (H) or for the percentages of Tle4 mRNA foci within a bin that were colocalized with 4E-T (I) or Pum2 (J). *p<0.05, **p<0.01 by one-way ANOVA with Dunnett's multiple comparisons test; n = 3 embryos each. (K) Confocal image showing FISH for Brn1 (red) and immunostaining of 4E-T (green) in the E16 VZ. The section was counterstained with Hoechst 33258 (blue). The boxed regions are expanded in the right panels, where the color channels are separated. Arrows denote Brn1-positive, 4E-T-positive foci, and arrowheads foci that are Brn1-positive only. (L, M) The E16 VZ/SVZ was divided into five bins of equal width and quantified for the percentage of total Brn1 mRNA-positive foci in each bin (L) or for the percentages of Brn1 mRNA-positive foci within a bin that were colocalized with 4E-T (M). *p<0.05, **p<0.01 by one-way ANOVA with Dunnett's multiple comparisons test; n = 3 embryos each. (N) Quantification of the percentages of Brn1 mRNA-positive foci in the apical-most bin (Bin 1) that were colocalized with 4E-T at E12 (white bar) and E16 (green bar). ***p<0.001; n=3 embryos each. Scale bars, 5µm. Error bars denote SEM.
Pum2 or 4E-T knockdown causes aberrant co-expression of Brn1 and Tle4 proteins during neurogenesis

These data suggest that a 4E-T/Pum2 complex selectively represses mRNAs to regulate cortical neurogenesis and neuronal specification. In this regard, we previously showed that 4E-T knockdown enhanced neurogenesis by derepressing bHLH proneurogenic mRNAs (Yang et al., 2014). Since our RIP data indicated that Ascl1, Neurog1 and Neurog2 were also Pum2 targets, we asked if Pum2 regulated neurogenesis by knocking it down with a previously-characterized Pum2 shRNA (Vessey et al, 2006; Vessey et al, 2010) after confirming its efficacy in cultured cortical precursors (Fig. 18A,B). Specifically, we electroporated E13/14 cortices with Pum2 shRNA and a nuclear EGFP plasmid, thereby selectively transducing RPs that generate predominantly (90%) superficial layer neurons (Tsui et al., 2013; Gallagher et al., 2015). Immunostaining three days later demonstrated that EGFP-positive cell locations were altered by Pum2 knockdown with a lower proportion in the VZ and CP, and a higher proportion in the SVZ (Fig. 11A,B).

We asked if these alterations reflected aberrant neurogenesis by immunostaining for EGFP and the RP marker Pax6, the proliferation marker Ki67, or the IP marker Tbr2 (Fig. 11C; Fig. 18C). Pum2 knockdown significantly decreased the proportions of EGFP-positive RPs and proliferating precursors, and increased EGFP-positive, Tbr2-positive IPs (Fig. 11D-F). These alterations were not due to increased cell death, since ≤ 3 EGFP-positive cells per section expressed the apoptotic marker cleaved caspase-3 two days post- electroporation with either control or Pum2 shRNA (n = 3 embryos each). Thus, like 4E-T knockdown, Pum2 knockdown enhanced neurogenesis.
To ask if Pum2 knockdown also affected neuronal specification, we performed similar electroporations and analyzed Brn1 and Tle4 protein expression. Immunostaining 3 days post-electroporation showed that in controls approximately 77% and 6% of EGFP-positive cells expressed Brn1 and Tle4 proteins, respectively (Fig. 11G-I). Pum2 knockdown had no effect on Brn1-positive cells, but significantly increased EGFP-positive, Tle4-positive cells by about 3-fold (Fig. 11H, I). Almost all EGFP-positive, Tle4-positive cells were also positive for Brn1 protein (Fig. 11G, J). The large majority of these triple-labelled cells were located outside the VZ with most in the intermediate zone or CP (Fig. 11G, J). Thus, Pum2 knockdown caused aberrant Tle4 protein expression in Brn1 protein-positive cells, predominantly superficial neurons.

We asked if 4E-T knockdown had similar effects using a previously-characterized 4E-T shRNA (Yang et al., 2014). Three days post-electroporation with either control or 4E-T shRNAs approximately 80% of EGFP-positive cells were Brn1-positive (Fig. 12A,B). However, the proportion of Tle4-positive cells was almost tripled by 4E-T knockdown, and almost all of these Tle4 protein-positive cells co-expressed Brn1 protein (Fig. 12A,C,D).

Several additional experiments argued that the aberrant co-expression of Tle4 in Brn1-positive cells was not simply due to enhanced neurogenesis. First, we performed similar electroporations with an expression plasmid for Creb binding protein (CBP) S436D, an activated CBP phosphomimic that enhances neurogenesis by regulating histone acetylation (Wang et al., 2010; 2012). As predicted, CBP S436D enhanced neurogenesis, as indicated by an increase in EGFP-positive cells in the CP and a decrease in the VZ (Fig. 12E). It did not, however, alter the proportion of EGFP-positive cells expressing Brn1 protein, Tle4 protein, or both (Fig. 12F,G). Second, we transfected cultured E11.5 cortical
precursors with Pum2, 4E-T or control shRNA and a nuclear EGFP plasmid. Immunostaining two days later showed that Pum2 or 4E-T knockdown increased the proportion of EGFP-positive, βIII-tubulin-negative precursors that co-expressed Tle4 and Brn1/2 proteins (Fig. 19A, B). Finally, we performed E13/14 electroporations with control, Pum2 or 4E-T shRNA, and analyzed them at 2 rather than 3 days, at which time point half of electroporated cells are RPs and a further 20-25% IPs (Yuzwa et al., 2016) (Fig. 19C,D). In controls, approximately 65-70% of EGFP-positive VZ/SVZ cells were Brn1 protein-positive and this did not change with Pum2 or 4E-T knockdown (Fig. 12H; Fig. 19E, F). In contrast, Pum2 or 4E-T knockdown caused an approximately 3-fold increase in EGFP-positive, Tle4 protein-positive VZ/SVZ cells, and almost all of these were also positive for Brn1 protein (Fig. 12I, J). Thus, Pum2 or 4E-T knockdown caused aberrant co-expression of Brn1 and Tle4 proteins in both precursors and newborn neurons.
Figure 11. Pum2 is important for regulating neurogenesis and neuronal specification. (Also see Fig. 18). E13/14 cortices were electroporated with a nuclear EGFP plasmid and Pum2 (shPum2) or control (shCtrl) shRNA, and coronal cortical sections were immunostained 3 days later at E16/17. (A) Representative images of electroporated sections immunostained for EGFP. Hatched white lines delineate the borders of the cortical regions. IZ = intermediate zone. (B) Quantification of images as in (A) for the percentages of EGFP-positive cells in each of the cortical regions. *p<0.05, **p<0.01; n=4 embryos each, 3-4 sections per embryo. (C) Representative confocal z-stack images of the VZ/SVZ of sections electroporated with Pum2 shRNA, and immunostained for EGFP (green) and Pax6 (red, top panels), Ki67 (red, middle panels) or Tbr2 (red, bottom panels). Arrows and arrowheads indicate EGFP-positive, marker-positive cells and EGFP-positive, marker-negative cells, respectively. (D-F) Quantification of sections as in (C) for the percentages of EGFP-positive cells expressing Pax6 (D), Ki67 (E) or Tbr2 (F). *p<0.05, **p<0.01; n=3-4 embryos, 3-5 sections per embryo. (G) Representative confocal images of electroporated sections immunostained for EGFP (green), Tle4 (red), and Brn1 (turquoise). Boxed regions are shown at higher magnification in the right panels, with the color channels shown individually. Nuclei are outlined with hatched white ovals, as defined by nuclear EGFP. (H-J) Quantification of sections as in (G) for the percentage of EGFP-positive cells expressing Brn1 (H), Tle4 (I), or both Brn1 and Tle4 (J) in either the entire cortex (Total Cortex) or in the SVZ, IZ and CP (Excluding VZ). ***p<0.001, ns = p>0.05; n=3 embryos each, 3 sections per embryo. Scale bars, 30µm in (A), 10µm in (C, G). Error bars denote SEM.
A) EGFP/Tle4/Brn1
- shCtrl
- sh4E-T

B) %Brn1+/EGFP+ Cells
- Control
- CBP Phosphomimic (S436D)

C) %Tle4+/EGFP+ Cells
- shCtrl
- sh4E-T

D) %Brn1+/Tle4+ /EGFP+
- shCtrl
- sh4E-T

E) %EGFP+ Cells
- VZ
- SVZ
- CP
- IS

F) %Brn1+/EGFP+ Cells
- VZ/SVZ

G) %Tle4+/EGFP+ Cells
- VZ/SVZ

H) %Brn1+/EGFP+ Cells
- VZ/SVZ

I) %Tle4+/EGFP+ Cells
- VZ/SVZ

J) %Brn1+/Tle4+ /EGFP+
- VZ/SVZ
**Figure 12. 4E-T regulates translation of Brn1 and Tle4 mRNAs during neurogenesis.** (Also see Fig. 19). (A) E13/14 cortices were coelectroporated with a nuclear EGFP plasmid and control (shCtrl) or 4E-T (sh4E-T) shRNA and coronal cortical sections were analyzed 3 days later by immunostaining for EGFP (green), Brn1 (turquoise), and Tle4 (red). Boxed regions are shown at higher magnification in the right panels, where the different color channels are separated. (B-D) Quantification of images as in (A) for the percentages of total EGFP-positive cells that were also positive for Brn1 (B), Tle4 (C), or both Brn1 and Tle4 (D). **p<0.01, ns = p>0.05; n=3 embryos each, 3 sections per embryo. (E-G) E13/14 cortices were coelectroporated with a nuclear EGFP plasmid and an expression construct for CBP S436D or the empty vector (Control) and coronal cortical sections were immunostained three days later for EGFP, Brn1 and Tle4. (E) Quantification of the percentage of EGFP-positive cells that were in each of the cortical regions. *p<0.05, **p<0.01, ns=p>0.05; n=3 embryos each, 2-3 sections per embryo. (F, G) Quantification of the percentages of total EGFP-positive cells that were positive for either Brn1 or Tle4 (F), or for both Brn1 and Tle4 (G). ns = >0.05; n=3 embryos each, 2-3 sections per embryo. (H-J) E13/14 cortices were coelectroporated with a nuclear EGFP plasmid and control (shCtrl), Pum2 (shPum2) or 4E-T (sh4E-T) shRNA and coronal cortical sections were analyzed 2 days later by immunostaining for EGFP, Brn1, and Tle4 (see Fig. 19E,F). Confocal images of these sections were then quantified for the percentages of total EGFP-positive cells in the VZ/SVZ that were also positive for Brn1 (H), Tle4 (I), or both Brn1 and Tle4 (J). **p<0.01, ***p<0.001, ns = p>0.05 by one-way ANOVA with Dunnett's multiple comparisons test; n=3 embryos each, 2 sections per embryo. Scale bars, 10µm. Error bars denote SEM.

**Disruption of Pum2 or 4E-T derepresses a deep layer neuron phenotype in newborn superficial layer neurons**

We next asked if the aberrant co-expression of Tle4 in Brn1-positive cells reflected a general derepression of a deep layer neuron phenotype by immunostaining electroporated sections for Brn1 and three other deep layer transcription factors, Ctip2, Tbr1 and FoxP2
(Fig. 13A,B; Fig. 20). In controls 72% of EGFP-positive cells were Brn1 protein-positive, and very few expressed Ctip2 (1%), Tbr1 (4-5%) or FoxP2 (6-7%) proteins (Fig. 13A-D,F,H; Fig. 20). Following Pum2 knockdown, EGFP-positive, Brn1 protein-positive cells were unaltered, but EGFP-positive cells expressing Ctip2, Tbr1 or FoxP2 were increased to more than 15% (Fig. 13A-D, F, H; Fig. 20), and almost all of these were also positive for Brn1 (Fig. 13E, G, I). These Brn1-positive, EGFP-positive cells aberrantly expressing deep layer transcription factors were almost all outside of the VZ, with many in the intermediate zone and CP (Fig. 13A-E). These cells were likely neurons, since only a small population of EGFP-positive, Ctip2-positive cells outside of the VZ expressed the IP marker Tbr2 (Fig. 13J).

We also asked about 4E-T knockdown. Similar electroporations showed that 4E-T knockdown increased the proportion of EGFP-positive, FoxP2 protein-positive cells from approximately 3-4% to about 15% (Fig. 13K, L) and that almost all of these FoxP2-positive electroporated cells were also positive for Brn1 (Fig. 13K, M). Thus, disruption of either Pum2 or 4E-T caused aberrant expression of a deep layer neuron phenotype in a subset of newborn Brn1-positive superficial neurons.
Figure 13. Pum2 and 4E-T are important for repressing a deep layer neuron phenotype in newborn superficial layer neurons. (Also see Fig. 20). (A-J) E13/14 cortices were electroporated with a nuclear EGFP plasmid and Pum2 (shPum2) or control (shCtrl) shRNA, and coronal cortical sections were immunostained 3 days later at E16/17. (A, B) Representative confocal images of electroporated sections immunostained for EGFP (green, both), Brn1 (red in A, turquoise in B), and either Ctip2 (turquoise, A) or Tbr1 (red, B). Boxed regions are shown at higher magnification in the right panels, with the color channels shown individually. Nuclei are outlined with hatched white ovals, as defined by nuclear EGFP. (C-G) Quantification of sections as in (A,B) for the percentages of EGFP-positive cells expressing Brn1 (C), Ctip2 (D), Brn1 and Ctip2 (E), Tbr1 (F), or Brn1 and Tbr1 (G) in either the entire cortex (Total Cortex) or in the SVZ, IZ and CP (Excluding VZ). *p<0.05, **p<0.01, ***p<0.001, ns = p>0.05; n=3 embryos, 3 sections per embryo. (H, I) Quantification of electroporated sections immunostained for EGFP, Brn1 and FoxP2 (see Fig 20) for the percentages of total EGFP-positive cells expressing FoxP2 (H) or Brn1 and FoxP2 (I). **p<0.01, ***p<0.001; n=3 embryos, 3 sections per embryo. (J) Representative confocal images of an electroporated section immunostained for EGFP (green), Tbr2 (red), and Ctip2 (turquoise). The boxed region is shown at higher magnification in the right panel, with the color channels shown individually. The nucleus of an EGFP-positive, Ctip2-positive, Tbr2-negative cell is outlined with the hatched white ovals, as defined by nuclear EGFP. (K-M) E13/14 cortices were electroporated with a nuclear EGFP plasmid and 4E-T (sh4E-T) or control (shCtrl) shRNA, and coronal cortical sections were analyzed 3 days later. (K) Representative confocal images of sections immunostained for EGFP (green), Brn1 (blue), and FoxP2 (red). Boxed regions are shown at higher magnification in the bottom panels, with the color channels shown individually. Nuclei are outlined with hatched white ovals, as defined by nuclear EGFP. (L, M) Quantification of sections as in (K) for the percentages of total EGFP-positive cells expressing FoxP2 (L), or Brn1 and FoxP2 (M). **p<0.01; n=3 embryos, 3 sections per embryo. Scale bars, 10µm. Error bars denote SEM.
4.4 Discussion

During embryogenesis, cortical RPs sequentially generate different neuronal subtypes with the earliest-born neurons populating deeper cortical layers and later-born neurons more superficial layers. Since it is now clear from lineage tracing studies that individual RPs generate multiple types of cortical neurons (Guo et al., 2013; Gao et al., 2014; Eckler et al., 2015), then a key question involves the molecular mechanisms determining this sequential neurogenesis. Several models could be invoked to explain these findings. In one extensively-investigated model, the genes that encode neuronal specification proteins are turned-on when a particular neuronal subtype is being generated, and then are rapidly turned-off when that subtype is no longer made (reviewed in Kwan et al., 2012; Greig et al., 2013). Here, we provide evidence for a second, not mutually exclusive, model where RPs are transcriptionally primed to make diverse cortical neuron subtypes, and post-transcriptional mechanisms select when and where neuronal specification mRNAs are translated.

The conclusion that RPs are transcriptionally primed to generate diverse cortical neurons comes from the scRNAseq and FISH analyses. These studies indicate that RPs co-express mRNAs encoding deep and superficial layer specification proteins throughout the neurogenic period. While there are fewer precursors co-expressing superficial and deep layer mRNAs at E17.5, the end of neurogenesis, there are nonetheless still many RPs and even newborn neurons with this mixed transcriptional phenotype. Precedent for this type of transcriptional priming comes from embryonic stem cells (Efroni et al., 2008), and makes biological sense from several perspectives. First, neurogenesis occurs within a short timeframe, and a switch from making one to another neuronal subtype could occur more rapidly if the mRNAs were already present and simply needed to be derepressed. Second,
transcriptional priming would allow fast extrinsic regulation of neuronal specification and thus provide flexibility within a rapidly-changing environment. Third, this model provides a mechanism for rapidly turning protein expression off, as exemplified by our data showing that in the absence of 4E-T and/or Pum2, deep layer specifiers are aberrantly translated during superficial layer neurogenesis. These studies do not preclude an important role for transcriptional regulation, but instead provide evidence for an additional regulatory layer that acts to ensure appropriate neuronal specification. These findings are also consistent with previous reports showing that (i) *Fezf2* mRNA persists in the VZ long after deep layer neurons have been generated (Guo et al., 2013), (ii) *Cux2* mRNA is expressed in the VZ before superficial layer neurons are made (Nieto et al., 2004; Guo et al., 2013), and (iii) *Ctip2* protein is only observed in postmitotic subcerebral neurons while *Ctip2* mRNA is expressed in cortical precursors (Leid et al., 2004; Arlotta et al., 2005).

Our findings raise a number of key questions. One of these involves the precise molecular nature of the translational repression complexes. In particular, our work defines a Pum2/4E-T complex that represses mRNAs regulating both the timing and specificity of neurogenesis (this study; Yang et al., 2014). However, many specification mRNAs that are expressed in RPs were not immunoprecipitated with Pum2 and thus are likely silenced by other RNA binding proteins and/or microRNAs. Moreover, Pum2 and 4E-T target mRNAs were only partially overlapping, indicating other protein partners for both of these translational repressors. In this regard, we recently showed that the RNA binding protein Smaug2 interacts with 4E-T in RPs to repress translation of the proneurogenic protein Nanos1 (Amadei et al., 2015).
A second key issue involves the association/dissociation of target mRNAs with Pum2/4E-T complexes. In this regard, both 4E-T and Pum2 are known phosphoproteins, Pumilio proteins are phosphorylated in response to growth factors like EGF (Kedde et al., 2010), and phosphorylation regulates Pumilio activity (Ota et al., 2011). Since embryonic cortical RPs are exposed to many growth factors that regulate neurogenesis (for example, see Yuzwa et al., 2016) then we propose that environmentally-driven signaling cascades directly regulate mRNA interactions with Pum2/4E-T complexes. However, simple phosphorylation-based models may not be sufficient to explain selective complex association with target mRNAs. For example, more than half of Brn1 mRNA but only 20% of Tle4 mRNA is complexed with 4E-T and Pum2 in E12 apical RPs. We believe that this selective association is likely mediated by other, as-yet-undefined proteins associating with Pum2, 4E-T and/or the mRNAs themselves, in agreement with recent data showing that many RNA binding proteins and components of the translational machinery are expressed and differentially regulated across cortical neurogenesis (DeBoer et al., 2013).

Our findings raise one final important question. How long does this transcriptional flexibility persist? Our data show that RPs continue to express neuronal specification mRNAs after neurogenesis is complete, and that some postnatal neurons express specification mRNAs for diverse neuronal phenotypes when they have already "chosen" a single identity at the protein level. These findings may thus reflect a general developmental flexibility with regard to neurogenesis and neuronal phenotypes, and may even provide a partial explanation for the ability to reprogram perinatal cortical neurons from one subtype to another with single transcription factors such as Fezf2 (Rouaux and Arlotta, 2013). This type of transcriptional priming may thus reflect a general cellular strategy where post-
transcriptional repression mechanisms provide an important way to ensure appropriate differentiation within a rapidly-evolving developing environment.
Figure 14 (related to Figure 6). Expression of neuronal specification mRNAs in embryonic RPs as determined by single cell transcriptome analysis. (A) scRNAseq data from E13.5, E15.5 or E17.5 cortical cells were analyzed and cortically-derived RPs, IPs, and neurons were identified by Yuzwa et al. (2017; GEO: GSE107122). Shown are t-SNE visualizations from all three embryonic timepoints, emphasizing the RP cell clusters (in red) that were analyzed in Figure 6. The numbers of cells in the RP clusters are also shown at each age. The IPs are color-coded in blue, and the neurons are in grey. (B) t-SNE visualizations of Brn1, Bhlhe22 and Tbr1, showing all cortically-derived cells at E13.5 and E15.5. Heat maps of the indicated gene are overlaid on the total cells in the RP clusters at each time point to show relative transcript levels in individual cells. Yellow denotes cells with no detectable transcript and blue/purple cells with the highest detectable transcript levels, as denoted by the adjacent color keys. Note that all three genes are expressed in cortical neurons.

Figure 15 (related to Figure 7). Controls for the FISH experiments. (A) Representative image of a coronal section through the E12 brain showing immunostaining for Brn1/2 (red), with counterstaining with Hoechst 33258 (blue). The boxed region in the cortex (Cx) is shown in the higher magnification images in Figure 7A. Note the positive Brn1/2
immunoreactivity in the midbrain. (B, C) Single molecule FISH analysis of E13 (B) and E16 (C) cortical sections hybridized to negative control probes tagged with red, green, or blue fluorophores, as supplied with the RNAscope kit (Advanced Cell Diagnostics). The different colors are pulled-apart for clarity. Sections were counterstained with Hoechst 33258 (light blue; upper left panels). LV = lateral ventricle. Scale bars, 500 µm in (A), 10µm in (B,C).

Figure 16 (related to Figure 8). Controls for the PLA experiments. Representative confocal z-stack images of E12 cortical precursors that were cultured for 3 days and analyzed by proximity ligation assays (gold dots) with antibodies for Pum2 and the NPY receptor (NPYR) or Pax6, and counterstained with Hoechst 33258 (blue, bottom panels). Scale bars, 5 µm.
Figure 17 (related to Figure 10). Controls for the colocalization experiments. (A) Representative z-stack confocal images showing FISH for Vcam1 and Aldoc mRNAs (white dots) in the E13 cortex. Sections were also counterstained with Hoechst 33258 (grey). The cortical regions are shown on the left. LV=lateral ventricle. (B) Representative confocal image showing FISH for Glo1 mRNA (red) combined with immunostaining for Pum2 (green) in the E12 cortical VZ. Sections were also counterstained with Hoechst 33258 (blue). Arrows and arrowheads denote Glo1 mRNA foci that are and are not colocalized with Pum2, respectively. The boxed regions are shown at higher magnification in the right panels, with the color channels shown individually. (C) The E12 VZ/SVZ was divided into five bins of identical width (see Fig. 9F) and quantified for the percentages of Glo1 mRNA foci in each bin that were colocalized with Pum2. n = 3 embryos. (D, E) Coronal sections through the E16 cortex were analyzed by immunostaining for Pum2 (red), 4E-T (green), Brn1 (green), Tle4 (red) and Ctip2 (green) (D), or by FISH for Brn1 mRNA, Tle4 mRNA or Ctip2 mRNA (white dots) (E). Sections were also immunostained for Pax6 to define the VZ, Tbr2 to define the SVZ and βIII-tubulin to define the CP (shown in D). In (E), the right panels show the Hoechst counterstain (grey) to visualize the cortical tissue. Shown are the VZ/SVZ and CP of the medial cortex. LV = lateral ventricle. Scale bars, 30µm in (A, D, E), 5µm in (B). Error bars denote SEM.
Figure 18 (related to Figure 11). Controls for the Pum2 knockdown experiments. (A) Representative images of cultured E12 cortical precursors that were transfected with EGFP and either control (shCtrl) or Pum2 (shPum2) shRNA, and immunostained 3 days later for EGFP (green) and Pum2 (red) and counterstained with Hoechst 33258 (blue). Arrows denote EGFP-positive cells and arrowheads denote EGFP-negative cells. (B) Quantification of images as in (A) for the percentage of EGFP-positive cells with readily-detectable Pum2 immunoreactivity. ***p<0.001; n = 3 experiments, 100 EGFP-positive cells per condition per experiment. (C) E13/14 cortices were coelectroporated with EGFP and control (shCtrl) or Pum2 (shPum2) shRNAs, and coronal sections were analyzed 3 days later. Shown are representative confocal z-stack images of the VZ/SVZ of sections electroporated with the control shRNA, and immunostained for EGFP (green) and Pax6, Ki67, or Tbr2 (all red). Arrows and arrowheads indicate EGFP-positive, marker-positive cells and EGFP-positive, marker-negative cells, respectively. Scale bars, 5µm in (A), 10µm in (C). Error bars denote SEM.
Figure 19 (related to Figure 12). Pum2 or 4E-T knockdown causes derepression of Brn1 and Tle4 mRNAs in cortical precursor cells. (A,B) Cultured E11.5 cortical precursor cells were transfected with an EGFP plasmid together with control (shCtrl), Pum2 (shPum2) or 4E-T (sh4E-T) shRNA, and two days later were immunostained for EGFP, Brn1/2, Tle4 and βIII-tubulin. These cultures were then quantified for the percentages of EGFP-positive, βIII-tubulin-negative precursor cells that expressed both Brn1/2 and Tle4 proteins. *p<0.05; n = 3 independent experiments. (C-F) E13/14 cortices were electroporated with a nuclear EGFP
plasmid and control (shCtrl), Pum2 (shPum2) or 4E-T (sh4E-T) shRNA and coronal cortical sections were analyzed 2 days later by immunostaining. (C, D) Quantification of the proportion of EGFP-positive cells in the different cortical regions. IZ = intermediate zone. *p<0.05; n=3 embryos, 2 sections per embryo. (E, F) Representative confocal images of electroporated cortical sections immunostained for EGFP (green), Brn1 (turquoise), and Tle4 (red). Boxed regions are shown at higher magnification in the right panels, where the different color channels are shown individually. Nuclei are outlined with hatched white ovals, as defined by nuclear EGFP. These sections were then quantified for the percentages of total EGFP-positive cells in the VZ/SVZ that were also positive for Brn1, Tle4, or both, as shown in Figure 12H-J. Scale bars, 10µm. Error bars denote SEM.

Figure 20 (related to Figure 13). Expression of FoxP2 protein following knockdown of Pum2 in the embryonic cortex. E13/14 cortices were electroporated with a nuclear EGFP plasmid and Pum2 (shPum2) or control (shCtrl) shRNA, and coronal cortical sections were immunostained 3 days later. Shown are representative confocal images of electroporated sections immunostained for EGFP (green), Brn1 (blue), and FoxP2 (red). Boxed regions are shown at higher magnification in the right panels, with the color channels shown individually. Nuclei are outlined with hatched white ovals, as defined by nuclear EGFP. Quantification of these images is shown in Figure 13H and I. CP=cortical plate; IZ=intermediate zone. Scale bars, 10µm.
Chapter 5. Translational repression regulates neural stem cell maintenance and differentiation in the postnatal brain

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Author Contributions

SKZ conceptualized, designed, performed, and analyzed most of the experiments and co-wrote the paper. SKZ and GY generated the 4E-T\textsuperscript{flo/flo} mice from cryopreserved mutant ES cells. MJB performed the scRNAseq experiments. DRK co-wrote the paper. FDM conceptualized experiments and co-wrote the paper.
5.1 Abstract

During embryonic development, the excitatory neurons that comprise the cortex are sequentially generated from neural precursor cells (NPCs). We have previously shown that these embryonic NPCs are transcriptionally primed to generate diverse types of neurons, and that a Pum2/4E-T translational inhibitory complex represses the translation of some of these neuronal identity mRNAs to ensure the appropriate temporal specification of daughter neurons (Zahr et al., 2018). Although it is well-established that a subpopulation of these embryonic NPCs persists postnatally and into adulthood within the ventricular-subventricular zone (V-SVZ), it remains largely unknown whether translational repression mechanisms regulate neural stem cell (NSC) maintenance and differentiation postnatally. To address this issue, we analyzed scRNAseq data from the early postnatal V-SVZ. This analysis revealed that postnatal NSCs are transcriptionally primed to generate progeny. Using single molecule fluorescence in situ hybridization (FISH) combined with immunohistochemistry (IHC), we show that proneurogenic mRNAs such as Ctip2 are expressed at the mRNA, but not protein level, within the postnatal V-SVZ. Conditional knockout of the translational repressor 4E-T postnatally leads to the aberrant expression of proneurogenic proteins, enhanced differentiation, and depletion of the NSC pool. Therefore, postnatal NPCs are transcriptionally primed to make neurons, but are maintained in an undifferentiated state via 4E-T mediated translational repression.
5.2 Introduction

During a narrow window of embryonic development, radial precursors (RPs) divide asymmetrically to generate the diverse excitatory neurons that populate the cortex. Subsequent to this wave of neurogenesis, which occurs between E11-17 in mice, the same pool of RPs switches to generating astrocytes and oligodendrocytes. Recent lineage-tracing studies have now shown that the postnatal and adult NSCs residing in the ventricular-subventricular zone (V-SVZ) surrounding the lateral ventricles emerge from a subpopulation of these embryonic RPs (Fuentealba et al., 2015; Furutachi et al., 2015). Unlike their embryonic predecessors, these NSCs primarily give rise to neuroblasts which migrate to the olfactory bulb (OB) and differentiate into GABAergic inhibitory interneurons (Lim and Buylla, 2014; 2016).

Previous work from our lab demonstrated that embryonic RPs are transcriptionally primed to generate excitatory neurons, but that a translational repression complex containing the 4E-T protein maintains them in an undifferentiated state by keeping mRNAs encoding proneurogenic proteins such as Neurog1/2 and NeuroD in a translationally repressed state (Yang et al., 2014). In subsequent work, we built on this model to show that embryonic RPs are transcriptionally primed to make diverse excitatory neuron subtypes, and that a Pum2-4E-T complex represses the translation of neuronal specifier mRNAs to ensure the appropriate temporal specification of daughter neurons (Chapter 4; Zahr et al., 2018). Although it is well-established that these embryonic RPs ultimately give rise to postnatal NSCs, it is largely unknown whether translational repression mechanisms continue to play a role in regulating NSC maintenance and differentiation in the postnatal brain (Fuentealba et al., 2015; Furutachi et al., 2015).
Here we have addressed this question and provide evidence that postnatal NSCs are transcriptionally primed to differentiate into progeny, but that 4E-T-mediated translational repression mechanisms maintain NSCs in an undifferentiated state in part via repression of proneurogenic mRNAs.

5.3 Results

**The 4E-T Translational Repressor is Expressed in Postnatal V-SVZ NSCs**

We first asked whether 4E-T is expressed in postnatal NPCs using two approaches. First, we immunostained postnatal day 2/3 (P2/3) V-SVZ sections for 4E-T. This analysis revealed immunodetectable 4E-T protein in most, if not all cells within the P2/3 V-SVZ niche (Figure 21A). Second, we combined single-molecule fluorescence *in situ* hybridization (FISH) for 4E-T mRNA with immunostaining for the pan-neural precursor marker Sox2 and neural stem cell (NSC) marker GFAP. 4E-T mRNA was detectably expressed in many GFAP-positive, Sox2-positive NSCs as well as GFAP-negative, Sox2-positive NPCs within the perinatal V-SVZ (Figure 21B). Therefore, 4E-T, the translational repressor we found played important roles embryonically, was abundantly expressed in NSCs of the early postnatal brain.
Figure 21. 4E-T is Expressed in Postnatal V-SVZ NSCs (A) Representative low-magnification image of the P2/3 ventricular-subventricular zone (V-SVZ) showing immunostaining for 4E-T (green, center and right). Sections were counterstained with Hoechst 33258 (dark blue) (B) Representative high-magnification confocal image of the P3 V-SVZ showing FISH for 4E-T mRNA (green, left and right images) and immunostaining for Sox2 (purple, center and right images) and GFAP (red, center and right images). The arrows and hatched ovals indicate Sox2-positive, GFAP-positive cells expressing 4E-T mRNA. The hatched white lines denote the border with the lateral ventricle (LV). Scale bars, 5µm.
Generation of a 4E-Tfl/fl mouse model

These data suggest that 4E-T mediated translational repression may play an important role in regulating NPC function at postnatal stages. To address this possibility, we generated a mouse carrying a floxed allele of the 4E-T gene (Figure 22A). The 4E-T floxed mice were generated from cryopreserved mutant C57BL/6 ES cells purchased from the Knockout Mouse Project. These ES cells have a 4E-T allele containing a lacZ trapping cassette (knockout-first allele) (Figure 22A) (Testa et al., 2004; Skarnes et al., 2011). Mutant ES cells were used to generate chimeric mice, which were subsequently crossed until the modified ES cells integrated into the germline. Mice that were heterozygous for the knockout-first 4E-T allele were subsequently crossed to mice expressing the flippase (FLP) recombinase (Figure 22A, B) (Wu et al., 2009). Flp-mediated recombination converted the knockout-first allele into a conditional allele containing loxP sites flanking critical exon 4 of 4E-T (4E-T\textsuperscript{fl}) (Figure 22A,C). 4E-T\textsuperscript{fl/+} mice were backcrossed to wild type (4E-T\textsuperscript{+/-}) mice for several generations to remove the FLP recombinase, and finally crossed to one another to generate 4E-T\textsuperscript{fl/fl} homozygotes (Figure 22C). Further details regarding generation of the mouse model are provided in the Materials and Methods section.
**Figure 22. Generation of the 4E-T conditional allele** (A) Schematic diagram of the 4E-T wild type (4E-T+), knockout-first (4E-Tkf), conditional (4E-Tfl), and conditional knockout (4E-Tcko) alleles. Boxes with numbers represent exons. The targeted knockout-first allele contains a lacZ trapping cassette and a neomycin cassette (blue boxes) inserted into the intron of the 4E-T gene, disrupting its function. The flippase (FLP) recombinase induces recombination of FLP recombinase target (FRT) sequences (green triangles), converting the knockout-first allele into a conditional allele (4E-Tfl). Cre recombinase induces

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recombination of loxP sites (red arrows), deleting the floxed exon to generate a frameshift mutation and trigger decay of the transcript. (B) PCR genotyping of wild type (*) and heterozygous 4E-T knockout-first alleles (**). Forward primer A and reverse primer C as shown in (A) were used. These primers produce a PCR product of 168bp for the 4E-Tkf allele and no band for the 4E-Twt allele. (C) PCR genotyping of wild-type (***) , heterozygous (**), and homozygous (*) 4E-T floxed alleles. Forward primer A and reverse primer B as shown in (A) were used. These primers produce a PCR product of 564bp for the 4E-Tfl allele and 358bp for the 4E-Twt allele. *, **, and *** indicate the 4E-Tfl/fl (homozygote), 4E-Tfl/+ (heterozygote), and 4E-T+/+ (wild-type) genotypes respectively. Band sizes in base pairs (bp) are indicated at the left-hand side in (B) and (C). Primer A=CSD-F, primer B=CSD-ttR, primer C=CAS_R1_Term (see Materials and Methods section for full sequences).

The 4E-T Translational Repressor Maintains Early Postnatal NPCs and inhibits Neurogenesis

To ask whether 4E-T regulated postnatal NPC maintenance and differentiation, we crossed the 4E-Tfl/fl mice to Nestin-CreERT2 transgenic mice. When the progeny of these mice are exposed to tamoxifen, 4E-T is selectively knocked out in Nestin-expressing NPCs. Newborn pups were exposed to tamoxifen via mother's milk between P1-P3, and the V-SVZ was analyzed at P8. We have previously shown that the Nestin-CreERT2 transgene induces recombination in almost all (~97%) Sox2-positive NPCs in the V-SVZ (Storer et al., 2018). Littermates that do not harbor the Nestin-CreERT2 transgene were used as controls in these experiments. To validate our knockout model, we performed FISH for 4E-T mRNA. This analysis revealed only background levels of 4E-T mRNA foci in Sox2-positive V-SVZ cells in NestinCreERT2+/−,4E-Tfl/fl (Cre+) compared to NestinCreERT2−/−,4E-Tfl/fl (Cre-) mice (Figure 23A). To ask if 4E-T deletion regulates NSC maintenance in vivo, we immunostained
P8 V-SVZ sections for Sox2 and GFAP to detect NSCs (Figure 23B). This analysis revealed that Sox2-positive, GFAP-positive NSCs were significantly reduced by 49% upon 4E-T deletion (Figure 23B and 23C). To ask if this decrease in early postnatal NSCs was due in part to reduced proliferation, we immunostained similar sections for Sox2, GFAP, and the proliferation marker Ki67 (Figure 23B). This analysis revealed a 50% reduction in triple-positive proliferating NSCs within the V-SVZ upon 4E-T depletion relative to controls (Figure 23B and 23D). As another measure of proliferation, we exposed newborn pups (P1-3) to tamoxifen, injected them with bromodeoxyuridine (BrdU) at P7, and immunostained sections 24 hours later for BrdU. This analysis revealed a ~48% reduction in total BrdU-positive cells upon 4E-T knockout (Figure 23E and 23F). These alterations were not due to increased cell death, since immunostaining similar sections for cleaved caspase 3 (CC3) revealed no significant differences in the number of CC3-positive cells between knockout brains and controls (Figure 23G). These data argue that 4E-T acts to maintain NSCs postnatally.

We next asked whether 4E-T ablation perturbed neuronal differentiation. In this regard, we have previously shown that 4E-T knockdown during embryonic development enhances neurogenesis by depressing mRNAs encoding proneurogenic proteins (Yang et al., 2014). To ask whether neurogenesis was altered by postnatal 4E-T ablation, we immunostained similar P8 V-SVZ sections for doublecortin (Dcx), a marker of GABAergic neuroblasts (Figure 23H). We then calculated the ratio of total Dcx-positive neuroblasts to total Sox2-positive, Gfap-positive NSCs, as a measure of neuronal differentiation. This analysis revealed a significant increase in the ratio of neuroblasts to NSCs upon 4E-T
conditional knockout, suggesting that 4E-T acts to maintain NSCs in part by preventing precocious differentiation (Figure 23I).
Figure 23. 4E-T Maintains Early Postnatal V-SVZ NSCs and inhibits Neurogenesis P1-3 NestinCreERT2^{−/+},4E-T^{fl/fl} (Cre−) and NestinCreERT2^{+/−},4E-T^{fl/fl} (Cre+) mice were exposed to tamoxifen via their mother’s milk, injected with BrdU at P7, and the V-SVZ was analyzed at P8. (A) Representative high-magnification confocal images showing FISH for 4E-T mRNA (red) and immunostaining for Sox2 (green). Nuclei are outlined with hatched white ovals. (B) Representative high-magnification confocal image showing immunostaining for Sox2 (green), Gfap (red), and Ki67 (purple). Arrows indicate triple positive cells. (C) Quantification of sections as in (B) for the total number of Sox2-positive, Gfap-positive cells (C) and Sox2-positive, Gfap-positive, Ki67-positive cells (D). **p<0.01; n=4 Cre−; n=5 Cre+ pups. (E) Representative high-magnification confocal image showing immunostaining for BrdU (red). (F) Quantification of sections as in (E) for total number of BrdU-positive cells. *p<0.05; n=3 pups each. (G) Quantification of sections for total number of CC3-positive cells. n=3 pups each. (H) Representative high-magnification confocal image showing immunostaining for Dcx (red). Arrows indicate Dcx-positive cells. (I, J) Quantification of sections as in (H) for the ratio of Dcx-positive cells to Sox2-positive, Gfap-positive cells (I) and total Dcx-positive cells (J). *p<0.05; n=5 pups each. The hatched white lines denote the border with the lateral ventricle (LV). Sections were counterstained with Hoechst 33258 (dark blue). Scale bars, 5µm. Error bars denote SEM.

Postnatal NSCs are Transcriptionally Primed to Generate Neurons

How does 4E-T maintain NSCs in an undifferentiated state? Our previous work has shown that embryonic RPs are transcriptionally primed to make neurons (Yang et al., 2014; Amadei et al., 2015; Zahr et al., 2018). Therefore, we asked whether postnatal NSCs are also transcriptionally primed to generate neuronal progeny. To address this possibility, we analyzed P6/P7 V-SVZ NSC single cell RNA sequencing (scRNA-seq) data generated elsewhere (unpublished data). We focused specifically on the NSC population, to ask about the expression of mRNAs encoding proteins shown to promote neurogenesis in the postnatal
brain, including *Ascl1, Pax6, Sp8, Sp9, Dlx1*, and *Dlx2* (Figure 24A-C) (Parras et al., 2004; Kohwi et al., 2005; Hack et al., 2005; Waclaw et al., 2006; Brill et al., 2008; Kim et al., 2011; Li et al., 2018). We observed that these transcription factors are expressed in 14-51% of NSCs (Figure 24C). Since the proneurogenic proteins that they encode are not detectable in NSCs (Doetsch et al., 2002; Hack et al., 2005; Waclaw et al., 2006; Parras et al., 2004; Brill et al., 2008; Kim et al., 2011; Li et al., 2018), then this suggests that a subpopulation of NSCs may be transcriptionally primed to generate neurons with translational repression playing an important role (Figure 24B,C) (Parras et al, 2004; Jang and Goldman, 2011).

To explore this idea that proneurogenic mRNAs are translationally repressed in postnatal V-SVZ NPCs, we focused on Ctip2, a transcription factor that is important for the genesis of GABAergic neurons (Arlotta et al., 2008). We combined FISH for *Ctip2* mRNA with immunostaining for Ctip2 protein on P2/3 sections (Figure 24D). We observed that Ctip2 protein was largely excluded from the V-SVZ, but that *Ctip2* mRNA was robustly expressed (Figure 24D). The *Ctip2* mRNA expression in V-SVZ NPCs was supported by our scRNAseq data, which showed that 37% of total V-SVZ cells express Ctip2 mRNA.
Figure 24. Postnatal NSCs are Transcriptionally Primed to Generate Neurons Analysis of scRNAseq data from P6/P7 V-SVZ NSCs. (A) t-SNE visualization of P6/P7 V-SVZ NSCs. (B) t-SNE visualizations of P6/P7 V-SVZ NSCs overlaid with the expression of Ascl1, Pax6, Ctip2, Sp8, Dlx2, Sp9, and Dlx1. (C) Table showing the percentages of P6/P7 NSCs expressing proneurogenic mRNAs. (D) Representative low-magnification image of P2/3 coronal sections showing immunostaining for Ctip2 (green). The boxed region of the V-SVZ
is depicted at higher magnification on the right, showing FISH for Ctip2 mRNA (red, center) and immunostaining for Ctip2 protein (green, right). Sections were counterstained with Hoechst 33258 (dark blue). (D) t-SNE visualization of the P7 V-SVZ scRNAseq data overlaid with expression of Ctip2 mRNA. For tSNE visualizations in (B), cells are color-coded according to expression level, ranging from not detected (yellow) to the highest detected levels (blue), according to the adjacent color key. Scale bars, 10µm.

**Disruption of the 4E-T complex Causes Aberrant Expression of Proneurogenic**

**Transcription Factors in NPCs**

These data show that postnatal NPCs express proneurogenic mRNAs in the absence of detectable protein, indicating that post-transcriptional regulation must be important. We therefore asked whether 4E-T represses the translation of proneurogenic mRNAs in postnatal NPCs. We exposed newborn pups to tamoxifen between P1-P3 to knockout 4E-T in Nestin-expressing NPCs, and analyzed the V-SVZ at P8 for the expression of the proneurogenic protein Ctip2 (Figure 25A). 4E-T conditional knockout led to a robust, 3-fold increase in the number of NPCs expressing the proneurogenic protein Ctip2 in postnatal NPCs (Figure 25B). This argued that 4E-T translationally repressed the proneurogenic transcription factor Ctip2 in NPCs.
Figure 25. 4E-T Conditional Knockout Causes Aberrant Expression of Proneurogenic Transcription Factor Ctip2 in NPCs P1-3 NestinCreERT2<sup>−/−</sup>;4E-T<sup>fl/fl</sup> (Cre-) and NestinCreERT2<sup>+/−</sup>;4E-T<sup>fl/fl</sup> (Cre+) mice were exposed to tamoxifen via their mother’s milk, and the V-SVZ was analyzed at P8. (A) Representative high-magnification confocal image showing immunostaining for Ctip2 (green). Arrows indicate Ctip2-positive cells. The hatched white lines denote the border with the lateral ventricle (LV). Sections were counterstained with Hoechst 33258 (dark blue). (B) Quantification of sections as in (A) for the total number of Ctip2-positive cells. **p<0.01; n=3 pups each. Scale bars, 5µm. Error bars denote SEM.

5.4 Discussion

Although extensive work has been done to identify the intracellular determinants of postnatal NSC maintenance and differentiation, the most well-studied mechanisms to date have focused on transcriptional and epigenetic control. However, our data support a model in which postnatal NPCs are transcriptionally primed to make neurons, but are maintained in an
undifferentiated state via 4E-T mediated translational repression. Disruption of 4E-T in postnatal NPCs leads to ectopic expression of proneurogenic specifiers, depletion of NSCs, and increased differentiation. In this model, extrinsic cues would cause dissociation of the 4E-T complex, leading to the translation of proneurogenic proteins and the rapid onset of neurogenesis. The ability to rapidly modulate protein expression levels is important in the early postnatal niche, where NPCs must integrate a multitude of cues to ensure the appropriate balance of proliferation versus differentiation. Indeed, the transition from NSC to activated TA cell involves extensive rearrangements in morphology and increased metabolic requirements, further highlighting the need for rapid control of protein synthesis.

These findings raise a number of major questions which should be explored in the future using our 4E-T floxed mouse model. How exactly does 4E-T maintain postnatal NPCs in an undifferentiated state? Based on our previous work, we hypothesized that the postnatal phenotype was in part due to derepression of proneurogenic mRNAs. In support of this hypothesis, we observed that Ctip2 mRNA and protein expression was discordant in the V-SVZ, and that knockdown of 4E-T led to ectopic Ctip2 protein expression. To gain further insight into how 4E-T acts to maintain NSCs, it is necessary to identify 4E-T mRNA targets postnatally using approaches such as RIP-seq. By overlapping postnatal and embryonic 4E-T targets, we would also be able to address (i) how 4E-T’s mRNA targets change over time, and (ii) whether there is a core set of mRNA targets that is maintained. Identifying these targets would also guide future investigation.

Does 4E-T mediated translational control regulate NSC cell fate decisions postnatally? This question stems from our earlier work showing that a Pum2/4E-T repressive complex regulates neuronal subtype specification embryonically (Zahr et al., 2018).
Postnatal/adult NSCs generate oligodendrocytes in addition to inhibitory interneurons, leading to the intriguing possibility that translational repression mechanisms occurring at the level of NSCs and/or their progeny regulate the decision to generate a neuron versus an oligodendrocyte (Menn et al., 2006). This idea is consistent with previous studies and our own work showing that Pax6 and Olig2, which specify neurons and oligodendrocytes respectively, (i) show discordant mRNA and protein expression in the V-SVZ, and (ii) are expressed in a mutually exclusive pattern at the protein level (Jang and Goldman, 2011; De Chevigny et al, 2012; unpublished observations). To investigate this possibility, postnatal targets identified by 4E-T RIP could be analyzed for the presence of transcription factors and/or receptors that specify distinct fates. Immunohistochemical analyses of 4E-T knockout brains for neuronal and gliogenic proteins could also be performed.

Our embryonic and postnatal work raises an important question: What are the upstream causes of association/dissociation of 4E-T inhibitory complexes from target mRNAs? 4E-T is a known phosphoprotein and can be phosphorylated by JNK in response to oxidative stress (Pyronnet et al., 2001; Caragnello et al., 2012). Given that postnatal NSCs are exposed to several extrinsic cues that regulate proliferation, differentiation, and cell fate specification, then we propose that environmentally driven signaling pathways regulate 4E-T interactions with target mRNAs (Yuzwa et al., 2016).

Several other questions remain that are apt for future investigation: What are the long-term effects of postnatal 4E-T depletion on olfactory bulb neurogenesis and interneuron subtype specification? What are the short- and long-term effects of 4E-T depletion on gliogenesis? Do 4E-T mediated translational repression mechanisms persist throughout adulthood? Is 4E-T similarly important in the hippocampal neurogenic niche, and are there
any behavioral effects of 4E-T depletion? In addition to NSCs, does 4E-T have cell-autonomous roles in NSC progeny such as TA cells, neuroblasts, and OPCs? Finally, what RBPs recruit 4E-T to its target mRNAs in the postnatal V-SVZ niche?
Chapter 6. Discussion and Future Directions

During embryogenesis, RPs sequentially give rise to diverse cortical neurons, followed by astrocytes and oligodendrocytes. A subpopulation of these RPs persists to become the postnatal V-SVZ NSCs which give rise to inhibitory interneurons and oligodendrocytes. Although the mechanisms instructing the genesis of these distinct progeny have been well-studied, most work to date has focused on transcriptional and epigenetic mechanisms. Many studies in non-mammalian model organisms have highlighted the importance of post-transcriptional regulatory mechanisms in stem cell development. Despite this work, the critical role of post-transcriptional control in mammalian neurogenesis has only recently begun to be appreciated. In my thesis, I have investigated the role of post-transcriptional regulation in both the embryonic and postnatal neurogenic niches, focusing on two translational repressors, 4E-T and Pum2. In Chapter 4, I showed that RPs are transcriptionally primed to make diverse excitatory neuron subtypes, and that a Pum2-4E-T complex represses the translation of neuronal specifier mRNAs to ensure the appropriate temporal specification of daughter neurons. In Chapter 5, I showed that 4E-T mediated translational repression mechanisms continue to regulate NSC maintenance and differentiation in the postnatal brain. These findings build on our understanding of how cell fate decisions are executed in a precise and rapid fashion during development. However, they also add another layer of complexity and raise important biological questions that should be explored in future work.
6.1 A Translational Repression Complex in Developing Mammalian Neural Stem Cells that Regulates Neuronal Specification

The work presented in chapter 4 support four main conclusions. First, RPs are transcriptionally primed to make diverse neuronal subtypes throughout embryonic neurogenesis. Second, the 4E-T repressor forms a complex with Pum2 during neurogenesis. Third, neuronal specification mRNAs are translationally repressed in RPs. Fourth, disruption of the Pum2/4E-T complex causes misspecification of cortical neurons.

The conclusion that RPs are transcriptionally primed to make diverse neuronal subtypes is based on our scRNAseq and FISH data showing that RPs co-express mRNAs encoding deep and superficial layer specification proteins throughout the neurogenic period. There is precedent for transcriptional priming in embryonic (ES) and hematopoietic stem cells (HSCs) and makes sense from several perspectives (Hu et al., 1997; Efroni et al., 2008). First, the genesis of diverse cortical layer neurons occurs within a relatively short time frame and switching from generating one type of neuron to another occurs much faster if the relevant mRNAs do not need to be transcribed de novo. Instead, they are already present in RPs and simply need to be derepressed. Second, translational repression offers a way of rapidly turning off protein expression, which would be necessary during the sequential genesis of distinct neuronal subtypes. This is highlighted by our finding that knockdown of Pum2 or 4E-T during superficial layer neurogenesis results in the aberrant translation of deep layer (subcerebral and corticothalamic) specifiers. In the absence of such a translational repression mechanism, deep layer mRNAs would continue to be translated until they degraded, a process that takes between 7.6-9hrs on average (Schwannhauser et al., 2011; Vogel et al., 2011). Third, transcriptional priming and translational repression enables rapid
regulation of neurogenesis and specification by extrinsic cues. This is critical in a developing niche in which several autocrine and paracrine interactions have been identified experimentally and/or predicted computationally (Gauthier-Fisher and Miller, 2013; Yuzwa et al., 2016; Voronova et al., 2017). In this regard, RPs respond to environmental cues from numerous sources such as newborn neurons, meninges, cerebrospinal fluid, and vasculature. One way in which these cues can be rapidly integrated to alter RP behavior is by triggering the association/dissociation of repressive complexes with target mRNAs.

Two recent studies lend support to our priming model. In one study, scRNAseq profiling of the developing cortex revealed that individual E13 and E15 RPs are remarkably similar at the transcriptional level (Yuzwa et al., 2017). In another study, murine E15 RPs transplanted into E12 cortices were able to generate earlier-born, deep layer neurons (Oberst et al., 2018). This finding can be readily explained if deep layer mRNAs are still expressed in E15 RPs, despite the fact that deep layer neurons are no longer being generated, and simply needed to be derepressed in response to cues present in the younger niche.

It is important to note that since high throughput methods such as Drop-seq are limited by relatively low sensitivity, the proportions calculated from our scRNAseq data are likely underestimates of the true level of specifier coexpression in RPs (Johnson and Walsh, 2017; Yuzwa et al., 2017; Zahr et al., 2018). Therefore, it would be worthwhile to perform a similar analysis using data derived from other methods with higher sensitivity such as 10X genomics (Johnson and Walsh, 2017). Nevertheless, we did observe that fewer RPs co-expressed deep and superficial layer specification mRNAs at E17.5 (at the end of the neurogenic period). We also observed a reduction in the average number of specification mRNAs expressed by individual RPs at E17.5 compared to E13.5 and E15.5. There are two
non-mutually exclusive explanations that may account for these observations. First, there
were fewer RPs profiled at E17.5, resulting in lower statistical power to detect lowly
expressed transcripts (233, 273, and 77 RP cells profiled at E13.5, E15.5, and E17.5,
respectively). Second, since RPs have switched to generating glia at this stage, they have
likely downregulated mRNA expression of cortical neuron specifier genes. At these later
stages, the promoters of cortical neuron specifiers may be epigenetically silenced and/or their
transcripts degraded by RBPs and/or miRNAs.

In line with the latter possibility, NPCs have been shown to undergo extensive
epigenetic changes during the formation of neurons, astrocytes, and oligodendrocytes (Yoon
et al., 2018). The incompetence of early NPCs to generating glia is attributed to
hypermethylation of gliogenic promoters such as glial fibrillary acidic protein (Gfap),
Olig1/2, and S100 calcium-binding protein B (S100b), which renders early NPCs
unresponsive to gliogenic cues such as BMP (Nakashima et al., 1999; Namihira et al., 2004;
Takizawa et al., 2001). A recent study using whole-genome bisulfite sequencing of Sox2-
GFP cells during embryonic cortical development showed that these glial promoters are
demethylated in late NPCs, allowing them to respond to gliogenic cues. Interestingly, this
demethylation of glial promoters is accompanied by de novo methylation and epigenetic
silencing of neural specific genes in gliogenic NPCs (Sanosaka et al., 2017). Based on these
observations, it is conceivable that the reduced expression of neuronal fate specifiers we
observe at E17.5 is due in part to epigenetic silencing. However, it is important to note that
since these aforementioned studies profile NPCs broadly, they do not only reflect the
epigenetic changes occurring within RPs.
How long does this transcriptional flexibility last with regard to cortical excitatory neuron specifiers? Interestingly, we observe a high level of mRNA expression of these excitatory neuron specifiers in postnatal V-SVZ NPCs when excitatory neurons are no longer being made under normal circumstances (unpublished observations). Based on this, it is tempting to speculate that postnatal NSCs, despite giving rise to inhibitory interneurons, retain excitatory neuron potential. Consistent with this idea, there is a body of evidence suggesting that excitatory neurons can be generated postnatally and in adulthood in the context of injury (Magavi et al., 2000; Chen et al., 2004). Following targeted apoptosis of layer VI corticothalamic and layer V corticospinal motor neurons in 6 week old adult mice, endogenous BrdU-labeled NPCs become neuroblasts that migrate into the injury site and differentiate into neurons with a pyramidal morphology, and appropriately project axons to the thalamus and the spinal cord respectively (Magavi et al., 2000; Chen et al., 2004). In another study, a subset of cells (~28%) from postnatal (P9-11) V-SVZ explants cultured individually on embryonic cortical slices give rise to glutamate-expressing neurons possessing a pyramidal morphology (Sequerra et al., 2010). Similar observations were made using explants from the 3 month old adult V-SVZ. These findings suggest that postnatal and adult V-SVZ NPCs retain the potential to generate excitatory neurons to a certain extent. However, it is important not to overinterpret the observation that embryonic excitatory neuron mRNAs are expressed in the postnatal V-SVZ niche, as it is likely that these transcription factors play different roles postnatally, such as in the genesis of GABAergic neurons (Arlotta et al., 2008; Brunjes and Osterberg, 2015; Nikouei et al., 2016).

The conclusion that neuronal specification mRNAs are translationally repressed in RPs comes from our FISH colocalization, RIP, and Pum2/4E-T knockdown data, and may
explain several observations from the literature. For instance, *Fezf2* mRNA expression (deep layer V neuron specifier) persists in the VZ after deep layer neurogenesis is complete, and *Cux2* mRNA expression (upper layer neuron specifier) precedes superficial layer neurogenesis (Nieto et al., 2004; Cubelos et al., 2010; Guo et al., 2013). Moreover, *Ctip2* mRNA (deep layer V neuron specifier) is expressed in NPCs while *Ctip2* protein is only detectable in postmitotic neurons (Leid et al., 2004; Arlotta et al., 2005). In light of our work, it is plausible that translational repression mechanisms ensure that these specifiers are expressed at the right time (early vs late RP) and/or cell type (RP vs neuron). In fact, *Ctip2* was one of the specifiers that was ectopically expressed upon Pum2 knockdown.

Another major finding from Chapter 4 is that even newborn neurons coexpress deep and superficial layer neuron mRNAs, despite being destined for a specific layer of the cortical plate. This is consistent with a recent scRNAseq study showing that late born (E14-15), 1-day old neurons (24 hrs after birth) coexpress superficial and deep layer markers. It is only by four days of age that neurons with a mutually exclusive expression of layer specific markers emerge (Telley et al., 2018). These findings argue that neuronal fate is progressively refined as RPs differentiate into neurons. This phenomenon is analogous to ES cell differentiation; pluripotent ES cells have higher global transcriptional activity as a consequence of their open-chromatin state, while loss of pluripotency and lineage specification is accompanied by globally reduced transcription as a result of epigenetic silencing of lineage specific genes (Meshorer, 2007; Meshorer et al., 2006; 2007; Efroni et al., 2008; Yoon et al., 2018). In light of our work, it is plausible that Pum2/4E-T-mediated translational repression mechanisms ensure that undifferentiated RPs do not prematurely commit to one lineage versus another despite being primed for multiple fates. However, as
particular neuronal subtypes mature and alternative lineages become epigenetically silenced; these translational repression mechanisms may no longer be as important for regulating cell fate. In mature neurons, these post-transcriptional regulators may switch to playing other important roles such as in axonal growth and synaptogenesis, processes that rely on local translation (Vessey et al., 2010; Sahoo et al., 2018).

Are embryonic RPs also transcriptionally primed to generate glia and/or inhibitory interneurons, and are the relevant mRNAs translationally repressed? Given that embryonic RPs are primed to generate diverse cortical neuron subtypes, it is possible that they are also primed to generate other types of progeny. In support of this possibility, the inhibitory interneuron specifier *Sp8* is both a Pum2 and 4E-T target according to our E12.5 RIP-Chip analyses (Waclaw et al., 2006; Yang et al., 2014; Zahr et al., 2018). To address these questions, we can analyze the expression of known GABAergic (e.g. *Dlx1/2, Sp8/9*) and gliogenic (*Olig1/2*) specifiers within embryonic RP scRNAseq clusters (Yuzwa et al., 2017). Moreover, Pum2 and 4E-T knockdown cortices could be analyzed for ectopic expression of glial and interneuron markers.

How do Pum2 and 4E-T silence target mRNAs in NPCs? As discussed in my introduction, Pum2 has been shown to repress target mRNAs in various ways. It can recruit the CCR4-NOT deadenylase complex leading to mRNA deadenylation and decay (Goldstrohm et al., 2006; 2007; Van Etten et al., 2012; Blewett and Goldstrohm, 2012; Weidmann et al., 2014). It can also interfere with translation by (i) antagonizing PABP’s translational activity, (ii) inhibiting translation elongation, and (iii) inhibiting translation initiation by interfering with eIF4E cap-binding (Cho et al., 2005; Cao et al., 2010; Friend et al., 2012; Weidmann et al., 2014). Similarly, 4E-T has been shown to repress translation
initiation by competing with eIF4G for binding to eIF4E, thus preventing the formation of the translation preinitiation complex (Dostie et al., 2000; Ferraiuolo et al., 2005). However, it has also been shown to destabilize mRNAs via recruitment of the CCR4-NOT deadenylase complex and decapping enzyme DDX6 (Kamenska et al., 2014; Ozgur et al., 2015; Nishimura et al., 2015). Our previous work has shown that, as in other cell types, 4E-T colocalizes with P-body components such as Dcp1, DDX6, and Lsm1, in NPCs (Dostie et al., 2000; Ferraiuolo et al., 2005; Yang et al., 2014). Furthermore, our proximity ligation assay data in Chapter 4 revealed that Pum2 interacts closely with 4E-T and the P-body component Dcp1. Consistent with this finding, a recent study found that Pum1/2 is enriched in purified P-bodies (Hubstenberger et al., 2017). The localization of Pum2 and 4E-T in P-bodies, however, does not distinguish between translational repression versus degradation, as both processes are thought to occur in P-bodies (Kiebler and Bassel., 2006; Balagopal and Parker, 2009).

Although we cannot distinguish between these silencing mechanisms on the basis of our data, a recent study suggests that Pum1/2 act primarily as translational repressors in the brain. The authors performed cross-linking immunoprecipitation (CLIP) of Pum1 and Pum2 in the brain, and analyzed a limited number of common Pum1/2 targets identified by CLIP using RT-qPCR and Western blot (Zhang et al., 2017). They found that, in most cases, target mRNA levels did not change upon Pum1 and/or Pum2 conditional knockout, but that their protein levels increased. These findings indicate that, at least in the brain, Pum1 and Pum2 may be acting primarily by repressing translation, with a secondary role in regulating the stability of a smaller subset of mRNAs (Zhang et al., 2017). To distinguish between these possibilities in the developing cortex, luciferase reporter assays using 3’UTRs of known
Pum2/4E-T targets could be performed in cultured NPCs. Performing qRT-PCR on the luciferase-3’UTR constructs following knockdown of Pum2 or 4E-T will indicate whether specific mRNA targets are also being degraded.

How does 4E-T fit within the larger landscape of post-transcriptional control? Our work supports a model in which 4E-T is recruited to different subsets of mRNAs via distinct sequence-specific RBPs (Yang et al., 2014; Amadei et al., 2015; Zahr et al., 2018). One such RBP is Smaug2, which interacts with 4E-T to repress the proneurogenic mRNA Nanos1 (Amadei et al., 2015). Notably, about 20% and 2% of 4E-T target mRNAs were associated with Pum2 and Smaug2, respectively (Zahr et al., 2018; Amadei, 2016), suggesting that several other RBPs and/or miRNAs are likely involved in targeting 4E-T to mRNAs. In this regard, one study showed that tristetraprolin, an RBP that interacts with AU-rich sequences in the 3’UTRs of target mRNAs, can recruit 4E-T to promote mRNA decay (Nishimura et al., 2015). 4E-T has also been shown to promote silencing of mRNAs targeted by miRNAs (Kamenska et al., 2014).

Our unpublished work further supports the idea that the association of 4E-T to its target mRNAs involves multiple interacting partners. Using a BioID approach in human neural precursor cells (hNPCs) derived from induced pluripotent stem cells (iPSCs), we identified several potential 4E-T interacting partners. We identified Argonaute proteins Ago1 and Ago2 (components of the RNA-induced silencing complex), suggesting that 4E-T can interface with miRNA silencing pathways in NPCs, as has been reported in HeLa cells (Kamenska et al., 2014). In addition, we identified various RBPs involved in various aspects mRNA metabolism (stability, translation, localization, and/or splicing) including Pum1, Pum2, Igf2bp1-3, Cpeb4, Mex3a, Csde1, Tra2b, among others (unpublished work). Taken
together, these findings point to a complex network of 4E-T dependent translational control. Future work should aim to validate these 4E-T interactions in developing RPs in vivo via coimmunoprecipitation and proximity ligation assays.

Despite the importance of 4E-T, several of our findings suggest that RBPs can act via 4E-T-independent mechanisms. First, mRNA targets of both Pum2 and Smaug2 only partially overlap with 4E-T (17% and 15% respectively) (Zahr et al., 2018; Amadei, 2016). Secondly, Pum2 and Smaug2 proteins do not completely colocalize with 4E-T-containing granules in NPCs (Zahr et al., 2018; Amadei, 2016). Third, if the function of RBPs were primarily dependent on 4E-T, we would predict that the 4E-T knockdown phenotype would be more severe than that of individual RBPs. However, the misspecification phenotype observed in vivo following Pum2 knockdown was of greater magnitude compared with that of 4E-T. Knockdown of Pum2 led to ectopic expression of a greater number of deep layer specifiers (Foxp2, Ctip2, Tbr1 in Pum2 knockdown vs Foxp2 in 4E-T knockdown). In addition, neurons failed to reach the cortical plate after Pum2, but not 4E-T, knockdown (Yang et al., 2014; Zahr et al., 2018). This mislocalization phenotype is consistent with the fact that “cell adhesion” and “migration” are among the most highly enriched gene ontology categories obtained from our analysis of Pum2 targets. Together, these observations strongly suggest that RBPs do not solely function via 4E-T dependent pathways.

How might Pum2 interact with target mRNAs in a 4E-T-independent fashion within RPs? Studies in both mammalian and non-mammalian model organisms point to several potential mechanisms. D. Melanogaster Pumilio can interfere with translation initiation by recruiting 4EHP, which in turn competes with eIF4E for binding to the mRNA cap (Cho et al., 2005). Alternatively, Xenopus Pum2 can compete directly with eIF4E for binding to the
5’ cap (Cao et al., 2010). Pum2 has also been shown to cooperate with miRNAs; miRNA binding sites are enriched near PREs in Pum1/2 targets, and Pum1/2 and PREs can enhance the translational repression of mRNAs by miRNAs targets (Galgano et al., 2008; Miles et al., 2012; Bohn., 2018). Therefore, RBPs such as Pum2 likely interact with target mRNAs via 4E-T independent mechanisms within RPs, likely via interactions with other miRNAs, RBPs, and/or translational inhibitors.

These observations suggest that although the Pum2-4E-T complex is clearly important, it is likely just the “tip of the iceberg” with regard to the relevant post-transcriptional machinery involved in RP cell fate choices. Indeed, many of the neuronal specification mRNAs that were expressed in embryonic RPs and neurons did not immunoprecipitate with either Pum2 or 4E-T, suggesting that additional RBPs and/or miRNAs may regulate them. Consistent with this idea, many RBPs and translational regulators have been shown to be expressed in a temporospatially dynamic manner throughout corticogenesis. In one study, DeBoer et al (2013) performed a microarray analysis at different timepoints throughout corticogenesis (at E11, E13, E15, and E18), specifically analyzing the expression of genes in post-transcriptional control associated GO categories such as mRNA splicing, localization, decay, stability, translation, and RNA binding proteins (DeBoer et al., 2013). This revealed that post-transcriptional regulatory factors are dynamically expressed across the neurogenic period, particularly those involved in translational control (DeBoer et al., 2013). A large number of RBPs were also dynamically expressed, and single-molecule FISH analyses of these RBPs found that their expression patterns were not only temporally dynamic but spatially specific in several cases (McKee et al., 2005; DeBoer et al., 2013).
Taken together, these findings point to an extensive network of post-transcriptional control in which 4E-T and Pum2 represent important nodes. In this model, post-transcriptional regulators control the expression of partially overlapping, functionally important, target mRNAs (Hogan et al., 2008). This partial redundancy provides the system more robustness and make sense from an evolutionary perspective, as perturbations in any one component would not lead to massive, irreversible alterations in gene expression (Felix and Wagner, 2008; Hogan et al., 2008). This model can be validated in future work by identifying the mRNA targets of RBPs expressed in the developing cortex, and determining the extent of overlap between them.

Previous work revealed that Pum2 forms a complex with the RBP Staufen2 in RPs, and that this complex segregates proneurogenic factors into differentiating daughter cells during asymmetric divisions (Vessey et al., 2012; Kusek et al., 2012). Our recent work has shown that Pum2 can also form a complex with 4E-T and P-body components to repress the translation of important mRNAs in RPs (Zahr et al., 2018). These findings raise the possibility that Pum2 can form complexes with distinct proteins for different purposes. In this model, the Pum2-Staufen2 complex would play a role in the asymmetric segregation of repressed mRNAs, while the Pum2-4E-T complex would be important for translational repression alone. Alternatively, Pum2, Staufen2, and 4E-T could be components of the same complex in RPs. In support of the latter possibility, Pum2, 4E-T, and D. Melanogaster Staufen have all been shown to colocalize in P-bodies (Ferraiuolo et al., 2005; Eulalio et al., 2007; Hubstenberger et al., 2017; Standardt and Weil, 2018). These two possibilities can be distinguished by determining whether Staufen2 and 4E-T interact using immunohistochemical analyses, coimmunoprecipitation, and proximity ligation assays.
Finally, our finding that Pum2 knockdown in vivo leads to misspecification of cortical neurons suggests an additional mechanism by which Pumilio loss of activity may contribute to neurological and neurodevelopmental disorders. A recent genetic study identified Pum1 deletions and missense mutations in patients with infantile- and adult-onset neurological disorders characterized by developmental delay, intellectual disability, seizures, and ataxia (Gennarino et al., 2018). Moreover, Pum2 loss of function has been shown to increase seizure propensity. These symptoms are usually attributed to Pum1/2’s activity in mature neurons, such as Pum2-mediated translational repression of voltage-gated sodium channels and GABA catabolic enzymes (Treiman, 2001; Driscoll et al., 2013; Kaplan et al., 2016; Follwaczny et al., 2017; Bohn et al., 2018). However, our data suggests that Pumilio loss of function can also impair earlier processes of neurogenesis and neuronal subtype specification. The incorrect numbers and/or types of neurons that result from this would lead to improper circuitry formation and may contribute to neurodevelopmental deficits, as has been demonstrated in a subset of children with Autism (Stoner et al., 2014).

In conclusion, the work presented in Chapter 4 argues that embryonic RPs co-express mRNAs encoding specifiers for multiple cortical neuron subtypes, and that a Pum2-4E-T complex represses the translation of some of these mRNAs to ensure the appropriate specification of daughter neurons. It is important to note that our findings do not discount the importance of transcriptional regulation in neuronal subtype specification, but add another regulatory layer that ensures the precise and timely genesis of distinct neuronal subtypes during cortical development.
6.2 Translational Repression Regulates Neural Stem Cell Maintenance and Differentiation in the Postnatal Brain

The work presented in chapter 5 supports the conclusion that postnatal V-SVZ NSCs are transcriptionally primed to make neurons, but maintained in an undifferentiated state via 4E-T mediated translational repression mechanisms. Disruption of 4E-T in postnatal NPCs leads to ectopic expression of proneurogenic specifiers, depletion of NSCs, and increases neuronal differentiation. The ability to rapidly modulate protein expression levels is important in the early postnatal niche, where NSCs must integrate a multitude of cues to ensure the appropriate balance of proliferation versus differentiation. In addition, NSCs are tasked with generating the correct types of progeny (i.e. neurons versus glia). As with embryonic RPs, these cell fate decisions not only require quickly turning on the expression of particular lineage programs, but rapidly turning off those that specify an alternative fate. Furthermore, the transition from NSC to TA cells and neuroblasts involves extensive rearrangements in morphology and increased metabolic requirements, further highlighting the need for rapid control of protein synthesis.

V-SVZ NSCs ultimately give rise to neuroblasts which migrate through the rostral migratory stream (RMS) to the olfactory bulb, where they complete their differentiation into different subtypes of interneurons (Bond et al., 2015; Lim and Buylla, 2014; 2016). Although the ratio of doublecortin-positive neuroblasts to Sox2-positive, GFAP-positive NSCs increased after 4E-T ablation, the total number of doublecortin-positive neuroblasts in the V-SVZ decreased. There are two possible interpretations of these findings. One possibility is that the acute increase in differentiation and concurrent depletion of NSCs results in a net reduction in the number of neuroblasts generated (i.e. fewer proliferative NSCs to generate
neuroblasts). Alternatively, more neuroblasts may have exited the V-SVZ and entered the RMS in the 4E-T knockout condition, given that they were generated prematurely. The latter possibility could be tested by quantifying the number of neuroblasts within the RMS in control and 4E-T conditional knockout sections. Irrespective of these possibilities, we predict that we may capture the acute increase in neuroblasts within the V-SVZ by analyzing an earlier timepoint following 4E-T ablation (e.g. analysis at ~P5).

Our initial findings raise a number of questions which should be explored in the future using our 4E-T floxed mouse model. How does 4E-T maintain postnatal NPCs in an undifferentiated state? Based on our previous embryonic work, we hypothesized that the postnatal phenotype was in part due to translational repression of proneurogenic proteins. In support of this, we observed that Ctip2 mRNA and Ctip2 protein expression were discordant in the V-SVZ, and that knockdown of 4E-T led to ectopic Ctip2 protein expression in NPCs. To gain a more complete understanding of how 4E-T acts to maintain NSCs, it is necessary to systematically identify 4E-T mRNA targets in the postnatal V-SVZ using approaches such as RIP-seq. Furthermore, by overlapping postnatal and embryonic 4E-T targets, we would also be able to address (i) how 4E-T’s mRNA targets change over time, and (ii) whether there is a core set of mRNA targets that is maintained.

What are the long-term effects of postnatal 4E-T knockout on olfactory bulb neurogenesis? To address this question, we could track the progeny of V-SVZ NPCs over a longer period of time (at least two weeks) using fluorescent reporters (e.g Rosa26-YFP) or BrdU-labelling following 4E-T ablation. The olfactory bulb could subsequently be analyzed for the number of interneurons marked by NeuN (mature neuron marker). Based on the
depletion of proliferative NSCs observed at P8/9, I predict that we would observe a long-term decrease in the newborn interneurons in the olfactory bulb.

As previously discussed, postnatal and adult V-SVZ NPCS retain the potential to generate cortical excitatory neurons to a certain extent (Magavi et al., 2000; Chen et al., 2004; Sequerra et al., 2010). Using our 4E-T floxed mouse model, we can now address whether these fates are translationally repressed in postnatal and adult V-SVZ NSCs. To do so, we can perform long-term experiments following 4E-T ablation and analyze the cortical layers for the presence of reporter-labelled neurons expressing excitatory neuron markers and possessing a pyramidal morphology. Moreover, given that postnatal V-SVZ NSCs give rise to oligodendrocytes that migrate to the corpus callosum where they function to myelinate axons, it will be important to investigate both the short- and long-term effects of 4E-T depletion on the genesis of oligodendrocyte progenitor cells (marked by Pdgfrα and Olig2) and mature oligodendrocytes (marked by MBP) (Menn et al., 2006; Xing et al., 2014).

In Chapter 4 we showed that a Pum2/4E-T repressive complex regulates neuronal subtype specification embryonically (Zahr et al., 2018). This work raises an important question: does 4E-T mediated translational control directly regulate NSC cell fate decisions postnatally? Postnatal/adult NSCs generate oligodendrocytes in addition to inhibitory interneurons, leading to the intriguing possibility that translational repression mechanisms occurring at the level of NSCs and/or their progeny regulate the decision to generate a neuron versus an oligodendrocyte (Menn et al., 2006). This idea is consistent with previous studies and our own work showing that Pax6 and Olig2, which specify neurons and oligodendrocytes respectively, show discordant mRNA and protein expression in the V-SVZ (De Chevigny et al, 2012). Moreover, Pax6 and Olig2 are expressed in a mutually exclusive
pattern at the protein level (<3% SVZ cells are Pax6-positive, Olig2-positive) (Jang and Goldman, 2011). In contrast, our own analysis of P6/P7 scRNAseq data suggests that Pax6 and Olig2 mRNA are coexpressed in a relatively larger proportion (~22%) of V-SVZ NSCs (unpublished observations).

These findings indicate that the decision to generate a neuron or oligodendrocyte may be regulated at the post-transcriptional level. To ask whether 4E-T-mediated repression is involved, several experimental approaches could be employed. First, a 4E-T RIP in the postnatal V-SVZ could be performed to determine whether specifiers of distinct fates are 4E-T targets. Second, any coexpression of neuronal and gliogenic markers in the V-SVZ following 4E-T conditional knockout could be analyzed via immunohistochemistry. Third, scRNAseq could be performed on the V-SVZ post-4E-T ablation. The latter approach would give us a global snapshot of how 4E-T depletion alters the proportions of and/or segregation between normally distinct cell types. It has the additional advantage of determining which transcripts become differentially expressed in various cell types upon 4E-T knockout.

As briefly mentioned in my introduction, neuroblasts can give rise to different subtypes of olfactory bulb interneurons such as superficial granule cells, deep granule cells, tyrosine hydroxylase-positive periglomerular cells, and calbindin-positive periglomerular cells (Lim and Buylla, 2014; 2016). This raises the interesting question of whether disrupting 4E-T-mediated translational repression interferes with interneuron subtype specification. This question can be readily tested using longer-term 4E-T conditional knockouts followed by immunohistochemistry of the olfactory bulb for different inhibitory interneuron markers.

Our work in Chapter 5 showing that 4E-T mediated translational repression is important for the maintenance of early postnatal NSCs raises the question of whether these
mechanisms persist throughout adulthood. Studies in adult V-SVZ NSCs and other stem cell compartments support this possibility. In this regard, hyperactivating mTORC1 in neonatal V-SVZ NSCs results in depletion of NSCs (i.e. reduced self-renewal) and increased differentiation into TA cells and neuroblasts (Hartman et al., 2013). On the other hand, reducing mTORC1 activity in NSCs prevents their differentiation and decreases neuroblast formation (Hartman et al., 2013). This phenotype was recapitulated in young adult mice in a follow-up study; elevating mTORC1 activity in slowly-dividing NSCs robustly increased TA cells and neuroblasts (Mahoney et al., 2016).

There is also precedent for translational control in other adult mammalian stem cell compartments. For instance, adult HSCs have less protein synthesis compared to their more committed progeny including common myeloid progenitors, granulocyte-monocyte progenitors, and other differentiated cell types. Interestingly, deletion of Pten (a negative regulator of mTOR signaling) in HSCs leads to activation of protein synthesis and exhaustion of HSCs (Signer et al., 2014). In an analogous fashion, translation increases during adult skin and muscle stem cell differentiation (Zismanov et al., 2016; Blanco et al., 2016). To ask about translational repression in the adult V-SVZ, we can use our NestinCreERT2;4E-T^{flo/flo} mouse model to conditionally knockout 4E-T in Nestin-expressing NPCs during adulthood and look for alterations, if any, in NSC maintenance, proliferation, and differentiation. Given the aforementioned studies in NSCs and other adult stem cell compartments, I predict that 4E-T will continue to be important for NSC maintenance and differentiation in the adult V-SVZ.

Given the importance of 4E-T in the V-SVZ niche, it would be interesting to investigate whether 4E-T plays a similarly important role in the subgranular zone (SGZ)
within the dentate gyrus of the hippocampus. In the SGZ, NSCs give rise to intermediate progenitor cells, neuroblasts, and ultimately, dentate granule neurons, and hippocampal neurogenesis has been implicated in cognitive processes such as learning and spatial memory (Toda et al., 2019). Much like the V-SVZ, most work on hippocampal neurogenesis has focused on transcriptional and epigenetic mechanisms. However, very recent work has implicated RBPs as critical regulators of hippocampal neurogenesis and development. In a recent study, the authors generated a Pum1 and 2 conditional knockout mouse and analyzed the effects of Pum1/2 knockout in Nestin-expressing cells on hippocampal neurogenesis in vitro and in vivo. Double knockout brains had a reduction in the volume of the dentate gyrus, coincident with an increase in Tbr2-positive intermediate progenitors, reduction in Dcx-positive immature neurons, and increased apoptosis of neurons. Consistent with these cellular deficits, these mice also exhibited learning and memory impairments (Zhang et al., 2017).

Using our 4E-T floxed mouse model, we can begin to ask about the role of translational repression in hippocampal SGZ. Given our work in Chapter 4 showing that Pum2 and 4E-T form a complex embryonically, I predict that 4E-T depletion in the hippocampal SGZ will similarly perturb NSC maintenance and differentiation. If this is the case, we could investigate the behavioral consequences of disrupted hippocampal neurogenesis using tests such as the Morris Water Maze (Christian et al., 2014).

It is important to emphasize one limitation of our transgenic mouse model. Although long considered to be a marker of NSCs broadly, Nestin is primarily expressed in actively dividing NSCs as opposed to quiescent NSCs (Codega et al., 2014). Therefore, the postnatal phenotype we observe using Nestin-CreERT2 likely reflects the effects of 4E-T depletion in actively dividing, rather than quiescent, NSCs. To circumvent this issue, pan-NSC-specific
transgenic lines such as Glast-CreERT2 could be used (Calzolari et al., 2015). Furthermore, we have observed that Nestin mRNA is highly expressed in multiple cell types within the V-SVZ in addition to dividing NSCs, such as transit amplifying cells, neuroblasts, and OPCs (observations from P7 scRNAseq data). Therefore, we cannot rule out cell-autonomous effects of 4E-T depletion in NSC progeny (TA cells, neuroblasts, and/or OPCs). Cell-autonomous roles in NSC progeny can be ruled out by directly knocking out 4E-T in neuroblasts (Dcx-CreERT2), and OPCs (Pdgfα-CreERT2) (Rivers et al., 2008; Zhang et al., 2010).

6.3 The Bigger Picture

Our embryonic (Chapter 4) and postnatal (Chapter 5) work raise several important questions. What are the upstream causes of association/dissociation of translational inhibitory complexes from target mRNAs? One way that translational repressors can be regulated is by post-translational modifications such as phosphorylation. The advantage of phosphorylation is that it provides a means of rapidly and reversibly altering the translation of subsets of mRNAs. In this regard, 4E-T is a known phosphoprotein and can be phosphorylated on six serine sites by JNK in response to oxidative stress, which triggers its accumulation into larger P-bodies (Pyronnet et al., 2001; Cargnello et al., 2012). It is also likely that Pum1/2 activity can be extensively modulated by post-translational modifications due to the presence of multiple phosphorylation sites (Goldstrohm et al., 2018). Despite this knowledge, the functional relevance of only one of these Pumilio sites is understood. Phosphorylation of Pum1 S714 in response to EGF stimulation has been shown to promote the binding and repression of CDKN1B/p27 mRNA, and in doing so, maintain cells in a cycling state (Kedde et al., 2010). Given that embryonic and postnatal NPCs are exposed to several growth factors
that regulate their proliferation, differentiation, and cell fate specification, then we propose that environmentally driven signaling pathways regulate Pum2/4E-T interactions with their target mRNAs (Yuzwa et al., 2016).

It is likely that other mechanisms in addition to phosphorylation regulate Pum2 and 4E-T activity. As described previously, both Pum2 and 4E-T act in combinatorial ways with other RBPs in a manner that alters their target specificity. In *D. Melanogaster*, Pumilio-mediated repression of *hunchback* mRNA is dependent on interactions with Brat and Nanos, while repression of *cyclinB* mRNA requires Nanos but not Brat (Wreden et al., 1997; Sonoda and Wharton, 1999; Sonoda and Wharton, 2001; Kadyrova et al., 2007). Furthermore, mammalian Pum2 and the RBP DazL cannot interact with *SDADI*’s 3’UTR, while Pum2 and the RBP BOL can (Urano et al., 2005). Mammalian Pum1/2 have also been found to act in a combinatorial fashion with other RBPs such as Nanos1-3, Trim 71 (Brat ortholog), and CPEB, in a manner which alters their regulation and target specificity (Pique et al., 2008; Campbell et al., 2012; Weidmann et al., 2016; Arvola et al., 2017). Similarly, both our work and that of others has shown that 4E-T homologs associate with several RBPs in addition to Pum2 such as Smaug2, CPEB, and OMA1/2 (Minshall et al., 2007; Standardt and Minshall, 2008; Sengupta et al., 2013). Taken together, these studies suggest that the specific mRNA targets that Pum2 and 4E-T associate with at any given stage of cortical development partly depends on the complement of RBPs present within an RP. This possibility is further supported by recent work showing that many RBPs are dynamically expressed during embryonic corticogenesis, and that a small subset of these RBPs play important functional roles (DeBoer et al., 2013; Pilaz and Silver, 2015; Popovitchenko and Rasin, 2017; Lennox et al., 2017).
Is transcriptional flexibility and translational repression a general cellular strategy employed in other developing mammalian stem cell compartments? As mentioned previously, translational control regulates mammalian stem cell maintenance in non-neural tissues (Signer et al., 2014; Blanco et al., 2016; Zismanov et al., 2016; Teixera and Lehmann, 2018). Additionally, there is evidence of transcriptional priming in ES cells and HSCs (Hu et al., 1997; Efroni et al., 2008). In this regard, multipotential HSCs coexpress erythroid and granulocytic lineage mRNAs prior to commitment to either an erythroid or granulocytic lineage, while ES cells coexpress mRNAs associated with ectodermal, endodermal, and mesodermal lineages (Hu et al., 1997; Efroni et al., 2008). These findings suggest that the decision to adopt a particular fate involves activating specific gene expression programs while silencing the expression of those that specify alternative fates. They also raise the possibility that choosing one fate over another is regulated in part by post-transcriptional regulatory processes. We can now address this possibility in other developing stem cell compartments using our 4E-T floxed mouse model.

Over millions of years of evolution, the cerebral cortex underwent a huge expansion in size and complexity, coinciding with the acquisition of higher cognitive abilities (Lui et al., 2011; Rakic, 2009; Sousa et al., 2017). Although cortical neurogenesis is highly conserved among mammals, there are distinct features that explain the expansion of NPCs and neuronal output in primates compared to rodents. One important feature is the expansion of an outer radial glial progenitor population in primates located in a region known as the outer SVZ. Like RPs, outer radial glia undergo several rounds of cell division, and in doing so increase neuronal output substantially (Fietz et al., 2010; Hansen et al., 2010; Reillo et al., 2011). Furthermore, the specific expansion of the human cortex is due, in part, to the fact that
human RPs undergo an increased number of self-renewing divisions compared to non-human primates and rodents (Lukaszewicz et al., 2005; Dehay and Kennedy, 2007; Geschwind and Rakic, 2013; Otani et al., 2016). Despite these differences, the molecular mechanisms underlying them are not fully understood. One explanation put forward is the emergence of novel-human specific duplicated genes. In this regard, a recent study identified 35 human-specific duplicated genes that were dynamically expressed during cortical neurogenesis. One of these was a paralog of the Notch receptor, NOTCH2NL, which robustly promoted NPC self-renewal via symmetric proliferative divisions, delayed neurogenesis, and increased long-term neuronal output (Suzuki et al., 2018; Fiddes et al., 2018).

Given the emerging roles of RBPs in murine corticogenesis discussed throughout this thesis, the increased expansion and complexity of the primate cortex may in part be explained by post-transcriptional mechanisms. In support of this idea, hundreds of miRNAs are expressed in the developing macaque but not mouse cortex, and a disproportionate amount of these primate-specific miRNAs are differentially expressed in the outer SVZ, where they regulate cell cycle and neurogenic genes (Arcila et al., 2014). Furthermore, all of the RBPs mentioned in this thesis have human and non-human primate paralogs, and our own analysis of recently published scRNAseq data from the developing human cortex revealed that these RBPs are highly expressed during the human neurogenic period (analysis of Zhong et al., 2018 data). It is therefore possible that RBPs play a role in primates to expand the progenitor pool and increase neuronal output. Furthermore, RBPs involved in alternative splicing have likely contributed to the increased complexity of human brain by amplifying the protein diversity of the transcriptome. In support of this idea, the human brain expresses more alternatively spliced mRNAs compared to other tissues (Johnson et al. 2009).
Strikingly, we observed that like murine RPs, individual human RPs are transcriptionally primed to differentiate into diverse neuronal subtypes (analysis of Zhong et al., 2018 data). Taken together, these findings suggest that analogous post-transcriptional mechanisms may be operating in the developing human cortex, and may partially underlie its increased expansion and complexity.


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## Table 1: 4E-T target mRNAs with computationally predicted Pum1/2 consensus binding motifs (related to Figure 9)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Symbol</th>
<th>Ensembl Gene</th>
<th>Pum2 RIP Identified</th>
</tr>
</thead>
<tbody>
<tr>
<td>17265451</td>
<td>Derl2</td>
<td>ENSMUSG00000018442</td>
<td></td>
</tr>
<tr>
<td>17310452</td>
<td>Golph3</td>
<td>ENSMUSG00000022200</td>
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<tr>
<td>17453106</td>
<td>Zfp11</td>
<td>ENSMUSG00000051034</td>
<td>Y</td>
</tr>
<tr>
<td>17307096</td>
<td>Zmym5</td>
<td>ENSMUSG00000040123</td>
<td></td>
</tr>
<tr>
<td>17387485</td>
<td>Zdhhc5</td>
<td>ENSMUSG00000034075</td>
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</tr>
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<td>17475685</td>
<td>Zfp60</td>
<td>ENSMUSG00000037640</td>
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</tr>
<tr>
<td>17350591</td>
<td>Sncap</td>
<td>ENSMUSG00000024534</td>
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</tr>
<tr>
<td>17431075</td>
<td>Arid1a</td>
<td>ENSMUSG00000007880</td>
<td>Y</td>
</tr>
<tr>
<td>17510823</td>
<td>Gab1</td>
<td>ENSMUSG00000031714</td>
<td>Y</td>
</tr>
<tr>
<td>17442269</td>
<td>Bcl7a</td>
<td>ENSMUSG00000029438</td>
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</tr>
<tr>
<td>17227570</td>
<td>Nek7</td>
<td>ENSMUSG00000026393</td>
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<tr>
<td>17514243</td>
<td>Irf2bp2</td>
<td>ENSMUSG00000051495</td>
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<td>17477151</td>
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<td>Bhlhe22</td>
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<tr>
<td>17220861</td>
<td>Slc30a1</td>
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<td>17469016</td>
<td>Wnt7a</td>
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<tr>
<td>17281582</td>
<td>Sos2</td>
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<td>17445755</td>
<td>Phtf2</td>
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<td>17520815</td>
<td>Ms12</td>
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<td>17493331</td>
<td>Pcf11</td>
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<td>17296537</td>
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<tr>
<td>17507014</td>
<td>Itgb1</td>
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<td>17426117</td>
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<tr>
<td>17394036</td>
<td>Fitm2</td>
<td>ENSMUSG00000048486</td>
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</table>

**Table 1. 4E-T target mRNAs with computationally predicted Pum1/2 consensus binding motifs (related to Figure 9).** A list of 4E-T target mRNAs (Yang et al., 2014) that had computationally-predicted Pum1/2 consensus sites. Computationally predicted targets that were identified by Pum2 RIP are marked as "Y." This table has been shortened. For the complete dataset, refer to the published supplementary materials corresponding to Chapter 4.
Table 2: Transcripts in the Pum2 target mRNA and background mRNA datasets (related to Figure 9)

<table>
<thead>
<tr>
<th>Probe ID</th>
<th>Gene Symbol</th>
<th>Ensembl Gene ID</th>
<th>Gene Accession</th>
<th>LFC</th>
<th>Adjusted p-value</th>
<th>Group</th>
</tr>
</thead>
<tbody>
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<td>17315178</td>
<td>Nr4a1</td>
<td>ENSMUSG0</td>
<td>NM_010444</td>
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<td>4.50E-05</td>
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</tr>
<tr>
<td>17286320</td>
<td>Foxq1</td>
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<td>NM_008239</td>
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<td>1.17E-04</td>
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</tr>
<tr>
<td>17519579</td>
<td>Gclc</td>
<td>ENSMUSG0</td>
<td>NM_010295</td>
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<td>4.53E-05</td>
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<td>Wrap73</td>
<td>ENSMUSG0</td>
<td>NM_021499</td>
<td>1.42586733</td>
<td>4.06E-05</td>
<td>target</td>
</tr>
<tr>
<td>17356583</td>
<td>Syvn1</td>
<td>ENSMUSG0</td>
<td>NM_028769</td>
<td>1.423716</td>
<td>4.15E-05</td>
<td>target</td>
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<tr>
<td>17293607</td>
<td>Ptc1</td>
<td>ENSMUSG0</td>
<td>NM_008957</td>
<td>1.41362733</td>
<td>4.06E-04</td>
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</tr>
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<td>17319989</td>
<td>Ldoc1l</td>
<td>ENSMUSG0</td>
<td>NM_177630</td>
<td>1.37554567</td>
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<tr>
<td>17530967</td>
<td>Slc38a3</td>
<td>ENSMUSG0</td>
<td>NM_023805</td>
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<td>17490551</td>
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</tr>
<tr>
<td>17241137</td>
<td>Unc5b</td>
<td>ENSMUSG0</td>
<td>NM_029770</td>
<td>1.33219567</td>
<td>1.18E-04</td>
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<tr>
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<td>17455401</td>
<td>Slc7a1</td>
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<td>NM_178070</td>
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<td>17347558</td>
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<td>NM_001033</td>
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<tr>
<td>17360440</td>
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<td>ENSMUSG0</td>
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<td>1.31028733</td>
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<td>17444372</td>
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<td>17309360</td>
<td>Slitrk1</td>
<td>ENSMUSG0</td>
<td>NM_199065</td>
<td>1.29125</td>
<td>4.69E-05</td>
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<tr>
<td>17389647</td>
<td>Rasgrp1</td>
<td>ENSMUSG0</td>
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<td>1.28603767</td>
<td>4.31E-03</td>
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<tr>
<td>17307522</td>
<td>Dleu7</td>
<td>ENSMUSG0</td>
<td>NM_173419</td>
<td>1.283474</td>
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<td>17483194</td>
<td>Sez6l2</td>
<td>ENSMUSG0</td>
<td>NM_144926</td>
<td>1.28009633</td>
<td>1.59E-04</td>
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<tr>
<td>17483325</td>
<td>Fbrs</td>
<td>ENSMUSG0</td>
<td>NM_010183</td>
<td>1.27160833</td>
<td>2.53E-04</td>
<td>target</td>
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<td>Irs2</td>
<td>ENSMUSG0</td>
<td>NM_0010812</td>
<td>1.26610233</td>
<td>2.27E-04</td>
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<tr>
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<td>ENSMUSG0</td>
<td>NM_009144</td>
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<td>1.62E-04</td>
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<tr>
<td>17371310</td>
<td>B3galt1</td>
<td>ENSMUSG0</td>
<td>ENSMUST00</td>
<td>1.25121667</td>
<td>3.09E-05</td>
<td>target</td>
</tr>
</tbody>
</table>

Table 2. Transcripts in the Pum2 target mRNA and background mRNA datasets (related to Figure 9) A list of mRNAs identified as Pum2 target mRNAs in the Pum2 RIP analyses, defined as the mRNAs that were at least 1.5-fold (p<0.05) enriched in the Pum2 immunoprecipitates relative to the total RNA input from mouse E12 cortex. The background dataset includes mRNAs that were not enriched in the Pum2 RIP (fold change of less than 1;
p<0.05) relative to input. This table has been shortened. For the complete dataset, refer to the published supplementary materials corresponding to Chapter 4.
Table 3: Gene ontology analysis of Pum2 target mRNAs and shared Pum2/4E-T target mRNAs (related to Figure 9)

<table>
<thead>
<tr>
<th>Pum2 Target List GO Terms</th>
<th>Count</th>
<th>P-Value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell adhesion</td>
<td>96</td>
<td>2.90E-15</td>
<td>Atp1b1,Cd24a,Cd9,Cd93,Dscaml1,Dscam,Epha2,…</td>
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<tr>
<td>cell migration</td>
<td>50</td>
<td>7.30E-13</td>
<td>Avl9,Brat1,Cd151,Cd248,Cd24a,Cdc42bpb…</td>
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<tr>
<td>positive regulation of transcription</td>
<td>93</td>
<td>1.10E-09</td>
<td>Aff1,Arid1a,Atmin,Cebpb,Ctf,Dp300,Ets2,E2f3,…</td>
</tr>
<tr>
<td>multicellular organism development</td>
<td>140</td>
<td>8.20E-09</td>
<td>Agfg1,Btbd7,Cnot1,Eid2,Epha7,Ephb3,Ephb4…</td>
</tr>
<tr>
<td>cell differentiation</td>
<td>109</td>
<td>1.20E-07</td>
<td>Agfg1,Cebpb,Ep300,Elf1,Elf4,Eid2,Epha2,Flt1…</td>
</tr>
<tr>
<td>neuron projection development</td>
<td>32</td>
<td>3.70E-07</td>
<td>Cd24a,L1cam,Mkl1,Mkl2,Pou4f1,Prdm12,Rab35…</td>
</tr>
<tr>
<td>cell cycle</td>
<td>84</td>
<td>8.90E-06</td>
<td>Bub3,Ctbp1,Chtf18,Ep300,E2f3,Fancd2,Fancl…</td>
</tr>
<tr>
<td>forebrain development</td>
<td>21</td>
<td>2.20E-05</td>
<td>Arid1a,Mkl1,Mkl2,Ndt1,Src,App,Aplp2,Apaf1,Arx…</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Joint Pum2/4E-T Target List GO Terms</th>
<th>Count</th>
<th>P-Value</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>regulation of transcription</td>
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<td>Arid1a,Bcl6,Ets2,E2f3,Gli2,Klf6,Mkl1,Meis1,Nkrf…</td>
</tr>
<tr>
<td>nervous system development</td>
<td>24</td>
<td>7.10E-09</td>
<td>Arid1a,Epha7,Sox1,Smarcd1,Ascl1,Ambra1,Chl1…</td>
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<tr>
<td>cell differentiation</td>
<td>29</td>
<td>9.70E-06</td>
<td>Gli2,Prdm16,Smurf1,Sox9,Ascl1,Adcyap1r1…</td>
</tr>
<tr>
<td>pattern specification process</td>
<td>8</td>
<td>2.00E-05</td>
<td>Gli2,Lfng,Ascl1,Hes1,Nog,Crkl,Zic1,Zic3</td>
</tr>
<tr>
<td>cell fate commitment</td>
<td>8</td>
<td>8.20E-05</td>
<td>Sox2,Sox9,Fgfr2,Gas1,Mef2c,Hes1,Neurog1…</td>
</tr>
<tr>
<td>neuron migration</td>
<td>9</td>
<td>4.50E-04</td>
<td>Mkl1,Sox1,Ascl1,Chl1,Cxcr4,Elp3,Met,Mef2c…</td>
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<tr>
<td>negative regulation of cell proliferation</td>
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<td>5.10E-04</td>
<td>Bcl6,Btg1,Sox9,Ambra1,Dll1,Fgfr2,Fgfr3,Itgb1…</td>
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<tr>
<td>stem cell population maintenance</td>
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<td>5.70E-04</td>
<td>Arid1a,Prdm16,Setd1a,Sox2,Eif4enif1,Fzd7,Stat3…</td>
</tr>
</tbody>
</table>

Table 3. Gene ontology analysis of Pum2 target mRNAs and shared Pum2/4E-T target mRNAs (related to Figure 9). A list of enriched gene ontology (GO) categories for Pum2 target mRNAs and shared Pum2/4E-T target mRNAs, as determined by DAVID. The number of genes, gene symbols, and p-values are included for each GO category. This table has been shortened. For the complete dataset, refer to the published supplementary materials corresponding to Chapter 4.
Table 4. PANTHER protein classifications of proteins encoded by shared Pum2/4E-T target mRNAs (related to Figure 9). A list of Pum2/4E-T target mRNAs and their corresponding protein classifications as determined by PANTHER. 126 of the 282 shared target mRNAs were assigned categories by PANTHER. This table has been shortened. For the complete dataset, refer to the published supplementary materials corresponding to Chapter 4.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Ensembl Gene ID</th>
<th>Species</th>
<th>Panther Protein Class</th>
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<tbody>
<tr>
<td>Stk17b</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>non-receptor serine/threonine protein kinase(PC00167)</td>
</tr>
<tr>
<td>Wars</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>aminoacyl-tRNA synthetase(PC00047)</td>
</tr>
<tr>
<td>Jun</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>basic leucine zipper transcription factor(PC00056);nucleic acid binding(PC00171)</td>
</tr>
<tr>
<td>Robo1</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td></td>
</tr>
<tr>
<td>Rcan1</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>signaling molecule(PC00207)</td>
</tr>
<tr>
<td>Fgd6</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>guanyl-nucleotide exchange factor(PC00113)</td>
</tr>
<tr>
<td>Ece1</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>metalloprotease(PC00153)</td>
</tr>
<tr>
<td>Urb2</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td></td>
</tr>
<tr>
<td>Spry1</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>signaling molecule(PC00207);transcription factor(PC00218)</td>
</tr>
<tr>
<td>Klf6</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td></td>
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<tr>
<td>Net1</td>
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<td>Mus musculus</td>
<td>receptor(PC00197)</td>
</tr>
<tr>
<td>Itgb8</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>cell adhesion molecule(PC00069);extracellular matrix glycoprotein(PC00100);receptor(PC00197)</td>
</tr>
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<td>Syt11</td>
<td>ENSMUSG0C</td>
<td>Mus musculus</td>
<td>membrane trafficking regulatory protein(PC00151)</td>
</tr>
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<td>Tacr3</td>
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</tr>
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<td>Mus musculus</td>
<td>G-protein coupled receptor(PC00021)</td>
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