Characterization of Cux2 in the Developing Cerebellum

by

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Abstract

A goal of developmental neurobiology is to understand the mechanisms involved in generating a mature nervous system from a thin multipotent neuroepithelium. For example, researchers are trying to elucidate the processes of progressive fate restriction, migration and maturation of neurons and glia in the cerebellum. Historically, the cerebellum has been studied in light of its important role in motor coordination, but more recently its role in a variety of other functions have been uncovered, highlighting it as an important brain region to investigate. The basic anatomy and connectivity of the adult cerebellum have been known for decades, but developmental biologists are only now beginning to uncover the origins of each cell type. Specifically, it is unclear whether progenitor cells in the two distinct germinal zones are fate restricted early in development or whether they are multipotent progenitors that become progressively fate restricted. Furthermore, the transcription factor Cux2 defines subsets of neural progenitors in multiple regions of the nervous system. Therefore, this study was aimed at investigating the role of Cux2 in the developing cerebellum. This study involved analyzing the expression pattern of Cux2 mRNA and protein throughout development to understand its restricted expression pattern. Subsequently, an inducible transgenic mouse line was used to label Cux2-expressing progenitor cells to delineate the fate of these progenitors. These experiments revealed that Cux2 activity was restricted to the developing rhombic lip and choroid plexus, and that these cells were fated to became mature granule cells. However, the final set of experiments suggests that *Cux2*, by itself, is not capable of altering the fate of cerebellar progenitors. These results continue to support the idea that Cux2 expression delineates a fate-restricted neural progenitor, and that within the developing cerebellum it defines a unique subset of progenitors in the rhombic lip that are restricted to a granule cell fate.

List of Abbreviations Used

bHLH	basic Helix-Loop-Helix
CP	Choroid Plexus
DCN	Deep Cerebellar Nuclei
E	Embryonic
GC	Granule Cell
GCL	Granule Cell Layer
HH	Hamburger-Hamilton
ISH	*In situ* Hybridization
MHO	Midbrain-Hindbrain Organizer
ML	Molecular Layer
NTZ	Nuclear Transitory Zone
PC	Purkinje Cell
PCL	Purkinje Cell Layer
PBS	Phosphate-Buffered Saline
PBT	PBS + Tween20
PFA	Paraformaldehyde
RL	Rhombic Lip
RP	Roof Plate
SVZ	Sub-Ventricular Zone
TBST	Tris Buffered Saline + Tween20
UBC	Unipolar Brush Cell
VZ	Ventricular Zone

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1 Corinthians 10:31
So whether you eat or drink, or whatever you do, do it all for the glory of God

Ephesians 6:7
Work with enthusiasm, as though you were working for the Lord rather than for people

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Psalm 28:7
The Lord is my strength and shield. I trust Him with all my heart.

Chapter 1: Introduction

A key question in developmental neurobiology concerns how progenitors progressively generate cell type identities. Understanding this process is not only of intrinsic interest to broaden our view of how the brain develops, but also has direct relevance to the mechanisms of disease processes that afflict the nervous system. In recent years developmental neurobiology has become even more focused on understanding the multitude of factors that play a role in maintaining proliferative cell populations, versus restricting them to particular lineages and terminally differentiating them into their mature, post-mitotic cell-types. Questions concerning these mechanisms are not only important for developmental biology, but have direct relevance to disease processes (Rossi and Cattaneo, 2002, Lindvall et al., 2004, Moody et al., 2013, Ozawa et al., 2016). For example, many pathways responsible for regulating apoptosis and cell survival during development have been implicated in cell loss during disease progression (Nijhawan et al., 2000). In addition, better understanding the processes that control proliferation will allow us to improve our methods to generate specific non-proliferating lineage restricted progenitor cells for cell replacement therapies (Rossi and Cattaneo, 2002, Lindvall et al., 2004, Moody et al., 2013). Therefore, studying developmental neurobiology will contribute to both a basic understanding of embryogenesis, as well as to the complex field of neurological disease.

Aberrant development of the cerebellum has been implicated in a plethora of childhood diseases, making it an important brain region on which to focus. For example, it is the site of medulloblastomas, the most common form of pediatric tumor. These tumors arise from a dysregulation of proliferative stem and progenitor cells. There are at least five categories of these tumors, with each category varying in response to therapies (de Bont et al., 2008, Hatten and Roussel, 2011, Roussel and Hatten, 2011, He et al., 2014, Ozawa et al., 2016). Each category of tumor arises by the dysregulation of a signaling pathway in a distinct cell population, such as Notch, Shh or BMP pathways, in proliferating granule cells (GCs) or ventricular zone (VZ) cells (de Bont et al., 2008, Hatten and Roussel, 2011, He et al., 2014). Furthermore, aberrant development of the cerebellum has been implicated in Autism spectrum disorders, highlighting its important role in cognitive functions. For example, vermal hypoplasia, altered reelin and glutamate receptor expression and a reduction in purkinje cell (PC) number, among other phenotypes, are common features in people with autism. Specifically, altered expression of factors such as glutamate and reelin have large and lasting effects on development, resulting in

aberrant cerebellar organization and function. These symptoms result in both motor and cognitive deficits, further highlighting the importance of the cerebellum in these processes (Fatemi, 2002, Fatemi et al., 2002, Fatemi et al., 2012). It is clear that improper cerebellar development can lead to a variety of developmental disorders. Although much research has gone into better understanding cerebellar development, it is essential that we continue to untangle the complex mechanisms that control proliferation and differentiation in this region in order to better understand these disorders.

1.1. The Cerebellum

The cerebellum has been studied for over a hundred years in large part because of its simple three-layered cortex, yet complex connectivity. As the name literally states, the cerebellum is the "little brain" and sits inferior to the cerebrum and posterior to the brainstem, forming the back wall of the 4th ventricle. Although historically the cerebellum was thought to only be involved in motor functions, recent evidence has highlighted its involvement in non-motor functions, including in cognition and emotional regulation, identifying it as a brain region potentially involved in neurodevelopmental disorders (Doyon et al., 2002, Bastian, 2006, Schmahmann and Caplan, 2006, Ito, 2008, Strick et al., 2009, Timmann et al., 2010).

The cerebellum is comprised of a few distinct cell types and has a complete circuit, making it an ideal model system for answering fundamental questions of development and organogenesis. The input to the cerebellum includes climbing fibers and mossy fibers. The inferior olive sends incoming climbing fibers that primarily synapse on PCs. In contrast, most of the afferents to the cerebellum are mossy fibers and primarily synapse on GCs. However, both climbing and mossy fibers project collaterals to deep cerebellar nuclei (DCN) (Eccles et al., 1966, Wu et al., 1999, Shinoda et al., 2000). The cortex is divided into three distinct layers. The deepest layer is the granule cell layer (GCL) and is made up predominantly of GCs, unipolar brush cells (UBCs) and Golgi cells. GCs, the most ubiquitous cell type, and UBCs both receive input from extra-cerebellar regions via excitatory mossy fibers and subsequently synapse with other GCs, UBCs, Golgi cells and PCs via ascending axons which bifurcate as parallel fibers in the molecular layer (ML) (Brodal and Drablos, 1963, Kunzle, 1975, Traynelis et al., 1993, Consalez and Hawkes, 2012, D'Angelo et al., 2013). The middle cortical layer is the purkinje cell layer (PCL) and is comprised of PCs and Bergmann glia. PCs have projections into the ML and

receive inputs from GCs, climbing fibers and interneurons (Konnerth et al., 1990, Lordkipanidze and Dunaevsky, 2005). The ML is the most superficial cortical layer and houses the projections of GCs and PCs, along with inhibitory interneurons, including stellate and basket cells (Consalez and Hawkes, 2012). The only output from the cerebellar cortex is from PCs, projecting to nuclei outside of the cerebellum and to DCN, which in turn form the rest of the cerebellar output (Ito and Yoshida, 1964, Ito et al., 1964, Arndt et al., 1998, Voogd and Glickstein, 1998). This complex connectivity and straightforward anatomy makes the cerebellum an excellent model for studying the mechanisms controlling proliferation and cell-type specification.

1.2. Neural Induction

Vertebrate development begins with the fertilization of an egg, resulting in a totipotent cell that can give rise to every cell type in an organism. Throughout embryogenesis this cell will proliferate and the embryo will develop polar axes, resulting in specialized regions and cell types (Hatten, 1999, Takaoka and Hamada, 2012, Moody et al., 2013). From early in development, organizing regions are critical patterning centers, beginning with the dorsal lip of the blastopore, which has the ability to pattern the entire newly developing embryo. This organizer contains many BMP and *Wnt* antagonists which are able to pattern adjacent tissues (Harland and Gerhart, 1997, Niehrs, 2004). As development progresses, the future neural tube must acquire neural identity by induction from the overlying midline of the dorsal-ectoderm (Lee et al., 1997, Sasai, 1998). Subsequently, by E9.5 in the mouse, the neural tube becomes regionalized into a posterior domain that will become the spinal cord and an anterior domain that will become the forebrain, midbrain and hindbrain (Liu and Joyner, 2001, Andoniadou and Martinez-Barbera, 2013, Benazeraf and Pourquie, 2013). These inductive phenomena during development are completed by a handful of main signaling pathways, including Notch, *Wnt*, *Shh* and retinoic acid, most of which function via paracrine signaling (Gerhart, 1999, Barolo and Posakony, 2002, Perrimon et al., 2012). The generation of a regionalized neural tube is orchestrated by the up- and down-regulation of these important signaling pathways at the proper time (Moody et al., 2013). For example, anterior neural induction is dependent upon the activation of *Fgf* signaling and concurrent inhibition of BMP, TGFβ and the *Wnt*/β-catenin pathways (Andoniadou and Martinez-Barbera, 2013). As development continues the diversity of cell types and signaling pathways continues to increase.

Early in development the neural tube is lined with proliferative neuroepithelial cells that will ultimately line the cerebral ventricular system and central canal of the spinal cord. However, before they become restricted neuroepithelial cells, they must increase in number, specialize in function and migrate to form distinct brain regions (Hatten, 1999). A variety of gradients, signaling mechanisms and transcription factors are required to maintain these cells in a proliferative state and to induce fate restriction and subsequent differentiation to create a brain that is regionally specified (Hatten, 1999, Takaoka and Hamada, 2012, Moody et al., 2013). For example, signaling proteins, such as *Wnt*s and *Fgf*s, are important in patterning tissue around the neural tube so that the brain can become an even more specified organ. Specifically, *Wnt1* is expressed in the midbrain, while *Fgf8* is expressed in the hindbrain, both of which are important in patterning this region. In addition, the discrete expression of these factors helps regulate the expression of other factors important in development and helps maintain the proliferative state of cells, while helping to specify the dorsal midbrain and hindbrain (Dickinson et al., 1994, Crossley et al., 1996, Danielian and McMahon, 1996, Liu et al., 1999, Martinez et al., 1999, Liu and Joyner, 2001, Rhinn and Brand, 2001, Green et al., 2014, Green and Wingate, 2014). This is only one example of the importance of signaling proteins in regulating proliferation and regionalization around the neural tube.

As the embryo continues to increase in size and complexity, the generation of cellular compartments is essential to ensure lineage restriction among certain cell types and to provide signaling to adjacent domains (Kiecker and Lumsden, 2005). The cerebellum is no exception to this developmental process as it is generated from a highly patterned region of the hindbrain through a multitude of complex signaling phenomena. For example, early in cerebellar development germinal zones, or compartments, must develop. These regions will give rise to specific cerebellar cell types as a result of their discrete microenvironments. Specifically, the rhombic lip (RL) will give rise to multiple types of excitatory neurons, as a result of its changing environment, lineage restriction of progenitors and compartmentalization (Martinez and Alvarado-Mallart, 1989, Alvarez Otero et al., 1993, Hallonet and Le Douarin, 1993, Hidalgo-Sanchez et al., 2005). It is clear that compartmentalizing the developing brain into discrete germinal regions is essential to give rise to a highly specialized brain.

1.3. Formation and Function of the Cerebellar Organizer

As mentioned previously, embryogenesis is driven by the generation of distinct organizing centers. Specifically, organizing centers direct proliferation and differentiation of adjacent tissue by secreting factors into the environment. One organizer in particular, the midbrain-hindbrain, or isthmic, organizer (MHO) is integral in the generation of the cerebellum and adjacent midbrain and hindbrain regions. Chick transplantation experiments revealed that this organizer region is able to regenerate and pattern hindbrain structures when grafted into a new organism (Hallonet et al., 1990, Hallonet and Le Douarin, 1993, Rhinn and Brand, 2001). Early in embryogenesis the isthmocerebellar primordium is both rostral and caudal to this isthmic region. It is this region, referred to as dorsal rhombomere 1, that will give rise to the cerebellum (Martinez and Alvarado-Mallart, 1989, Alvarez Otero et al., 1993, Hallonet and Le Douarin, 1993, Hidalgo-Sanchez et al., 2005). Throughout early development a variety of factors are expressed around the MHO, including both transcription factors, such as Engrailed (*En*), *Pax*, *Otx* and *Gbx* families, and secreted factors, such as *Wnt* and *Fgf* (Dahmane and Ruiz i Altaba, 1999, Rhinn and Brand, 2001, Hidalgo-Sanchez et al., 2005). These factors all play important and specific roles in maintaining this organizer region and inducing subsequent neurogenesis.

A fine-tuned combination of all of these signaling factors is important for proper development of the MHO. One of the initial steps in the generation of this region is the establishment of anterior *Otx2*- and posterior *Gbx2*-positive domains. This process occurs at embryonic (E) day 7 in the mouse and Hamburger-Hamilton (HH) stage 4 in the chick. It is postulated that the anterior mesoderm or notochord regulates *Otx2* expression rostral to the organizer while, at the same time, *Gbx*2 expression is restricted caudally (Rhinn and Brand, 2001, Hidalgo-Sanchez et al., 2005). Although the expression of *Otx2* and *Gbx2* arise independently, they become dependent on each other for expression maintenance and subsequent boundary formation, highlighting the complex interplay between these developmental regulators (Millet et al., 1996, Millet et al., 1999, Rhinn and Brand, 2001). If these genes are lost it results in aberrant development of surrounding regions. In addition, if they are ectopically expressed it results in an expansion of the organizer region. For example, ectopic caudal expansion of *Otx2* expression causes the organizer to move more caudally. In contrast, the rostral expansion of *Gbx2* expression results in a more rostral organizer region (Millet et al., 1996, Millet et al., 1999, Rhinn and Brand, 2001, Hidalgo-Sanchez et al., 2005). Once the border region becomes more

established it activates *Pax2* signaling, and subsequently *En1, Wnt1* and *Fgf8* signaling (Rhinn and Brand, 2001, Hidalgo-Sanchez et al., 2005). The development of this isthmic border defines the initial distinction of the MHO.

The establishment and maintenance of the MHO is dependent on *Wnt1* and *Fgf8* signaling. For example, *Wnt1* begins being excreted in the *Otx2*-positive domain and is important for the maintenance of this boundary region, but is not sufficient on its own to induce patterning in this region (Dickinson et al., 1994, Liu and Joyner, 2001). In contrast, *Fgf8* is able to induce the expression pattern typical of the MHO, identifying it as an important factor in organizer generation (Crossley et al., 1996, Liu et al., 1999, Martinez et al., 1999, Rhinn and Brand, 2001). Furthermore, if expressed ectopically in the developing chick midbrain, *Fgf8* is able to shift the midbrain-hindbrain border more rostral. Specifically, *Fgf8* helps regulate the proliferative state of cells and maintain the expression of other key factors, such as *Math1*, a gene involved in fate determination in the cerebellum (Green et al., 2014). It is clear that orchestrated expression and restriction of secreted factors are both important for boundary maintenance, and are regulated in part by *Wnt1* and *Fgf8* expression.

In addition, the homeobox transcription factors *En1* and *En2*, along with *EphA4* and *EphA5*, are important for patterning the midbrain-hindbrain junction. *En2* begins being expressed early in development and is maintained by midbrain-secreted *Wnt* (Danielian and McMahon, 1996, Dahmane and Ruiz i Altaba, 1999). *En2* expression, along with its paralog *En1*, is strongest at the midbrain-hindbrain junction and decreases in both the rostral and caudal direction from there (Millet and Alvarado-Mallart, 1995, Dahmane and Ruiz i Altaba, 1999, Hidalgo-Sanchez et al., 2005). Although this general pattern of expression is maintained throughout development, these factors become increasingly restricted to the midbrain-hindbrain junction (Dahmane and Ruiz i Altaba, 1999). Similarly, early in development Ephrin receptor and ligand expression are important in establishing hindbrain borders. *EphA4* expression increases in the caudal direction during hindbrain morphogenesis and cerebellar development. The expression of this factor is restricted to the developing cerebellum and a small neighboring region of the isthmus and is not expressed in more ventral regions or in the rest of the isthmus. Interestingly, *EphA5* expression is complementary to that of *EphA4*, as it is most highly expressed in the ventral hindbrain and in the isthmus and decreases into the cerebellar anlage (Karam et al., 2000). As hindbrain development continues, an increasing number of factors

become important for regionalization of the newly forming anlage. This early pattern generation also highlights the importance of discrete spatiotemporal expression of factors during neural development.

1.4. Formation of the Rhombic Lip Region

As cerebellar development continues, the anlage becomes divided up into two distinct germinal zones that will give rise to all of the cell types of the mature cerebellum (Fig. 1). Around E9 in the mouse these two germinal zones become anatomically distinct. These regions derive from the metencephalon and are the RL and VZ over the developing 4th ventricle (Hallonet et al., 1990, Wingate and Hatten, 1999, Hidalgo-Sanchez et al., 2005). The cells around the neural tube become increasingly restricted in their potential to give rise to these two tissues during early development. For example, at E2 in the chick (HH stage 11-13) the dorsal two-thirds of the neural tube can contribute to the RL. However, by E5 (HH stage 25-28) it is only the most dorsal region of the neural tube that can give rise to RL cells (Wingate and Hatten, 1999). The generation of the RL is completely dependent on *Math1* function and partially dependent on *Zic1* function (Ben-Arie et al., 1997, Machold and Fishell, 2005, Wang et al., 2005). The earliest born cells from the RL migrate to form nuclei outside of the cerebellum. The next population of cells generated from the RL become projection neurons of the DCN (Green and Wingate, 2014). Finally, the last two cell types generated from the RL are GCs and UBCs (Hallonet et al., 1990, Wingate and Hatten, 1999). In contrast, the VZ contains multipotent progenitors that will give rise to all PCs, inhibitory interneurons and glia at overlapping time points (Lin and Cepko, 1999, Wang and Zoghbi, 2001, Martinez et al., 2013). Both of these regions will give rise to every cell type within the cerebellum, requiring strict spatiotemporal expression of a variety of factors to orchestrate this complex process.

In order for these regions to become more distinct and for the developing cerebellar anlage to continue to grow out from the hindbrain, both the RL and VZ must proliferate extensively. Perhaps not surprisingly, *Shh* is important during this phase of cerebellar development, playing an integral role in proliferation and differentiation of VZ and RL derivatives (Dahmane and Ruiz i Altaba, 1999, Huang et al., 2010). As a secreted factor, *Shh* can be found in the circulating cerebrospinal fluid during development. This allows it to signal with

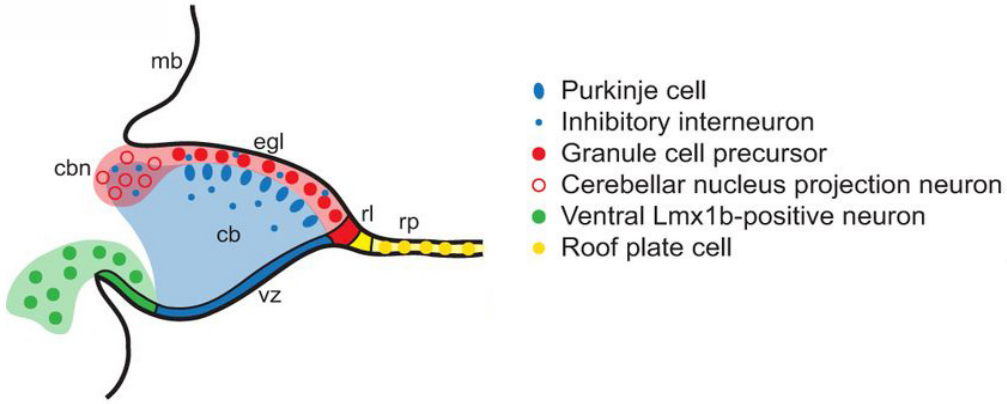

Figure 1. An overview of cerebellar development showing the RL that will give rise to all excitatory neurons and the VZ that will give rise to all inhibitory neurons and glia. rl= rhombic lip; vz= ventricular zone; mb= midbrain; cbn=cerebellar nucleus; egl= external granular layer; rp= roof plate; cb= cerebellum. Used with permission (Butts et al., 2014).

the VZ radial glial progenitors lining the fourth ventricle beginning at E13.5. Surprisingly, *Shh* isn't found in the developing cerebellum until E16.5 (Huang et al., 2010). Furthermore, it has been shown that receptor mutations, an up-regulation or other aberrant functions of this signaling pathway, can result in the genesis of medulloblastomas (Goodrich et al., 1997, Dahmane and Ruiz i Altaba, 1999, Romer et al., 2004, Hatten and Roussel, 2011). In addition, if chick embryos are injected with hybridoma cells that block *Shh* signaling, it results in severe cerebellar defects, including thinner and disorganized layers and folia (Dahmane and Ruiz i Altaba, 1999). Clearly, *Shh* signaling is important for the initial patterning, proliferation and cell type specification of most cerebellar cell types during development.

1.5. Mediolateral Organization of the Cerebellum

Even after the complex generation of all of the neuronal and glial populations, cells must successfully mature into their respective post-mitotic cell type and establish the stereotypical organization of the adult cerebellum. The cerebellum is organized into multiple "stripes", or functional domains, that run as parasagittal stripes from medial to lateral. These units and boundaries are established by heterogeneous expression of a variety of factors, such as Zebrin II and phospholipase C (Demilly et al., 2011, Oberdick and Sillitoe, 2011, Consalez and Hawkes, 2012, White and Sillitoe, 2013). Furthermore, the cerebellum must develop the proper number and organization of folia, a complex process that is essential for the development of the specific and specialized motor circuits of this region (Sotelo, 2004, Sillitoe and Joyner, 2007, Sudarov and Joyner, 2007, Yopak et al., 2007). Therefore, during development the cerebellum has many complex phenomena to perform, including the generation of multiple distinct cell types, the migration and maturation of these cells and the establishment of the classical cerebellar organization. All of these processes rely on complex molecular patterning and signaling factors.

1.6. Cerebellar Ventricular Zone Derivatives

The VZ gives rise to all of the inhibitory neurons and glia of the cerebellum, including PCs, GABAergic interneurons and astrocytes. PCs are the most anatomically obvious cell type in the cerebellum, with very large soma and arborescent dendrites that reach into the ML, synapsing with GCs and interneurons. In addition, since most interneurons in the cerebellum are inhibitory, they are almost all VZ derivatives that migrate throughout the cerebellum (Hoshino et al., 2005,

Lundell et al., 2009, Consalez and Hawkes, 2012, Leto et al., 2012). Finally, most glia are generated from the VZ (Dahmane and Ruiz i Altaba, 1999). Since the VZ is responsible for generating such a diverse population of cells from a common pool of progenitors, spatiotemporal restriction of transcription and signaling factors is imperative to generate molecularly distinct precursor populations.

One very important factor during neurogenesis in the VZ is *Ptf1a*, a bHLH (basic Helix-Loop-Helix) transcription factor. *Ptf1a* is essential for the generation and differentiation of progenitors into both GABAergic neurons and glia (Hoshino et al., 2005, Pascual et al., 2007, Leto et al., 2012). For example, animals that lack *Ptf1a* expression do not generate GABAergic cerebellar neurons, including interneurons or PCs, resulting in the absence of the entire cerebellar cortex (Hoshino et al., 2005). Instead, VZ cells in these animals migrate into the External Granular Layer (EGL) and exhibit granule cell-like phenotypes (Pascual et al., 2007). If *Ptf1a* expression is induced in glutamatergic neuronal precursors it results in the switch to GABAergic neuron production. Interestingly, the inhibitory neurons that are produced exhibit neurotransmitter, morphology and migration phenotypes reminiscent of GABAergic neurons (Hoshino et al., 2005). These results highlight the importance of *Ptf1a* expression in the VZ and its role in the decision of cells to adopt either a GABAergic or glutamatergic phenotype. These studies, among others, have revealed common factors among all of the VZ derivatives, leading to an initial thought that VZ progenitors consisted of a homogenous population.

However, although it was initially thought that all VZ derivatives arise from a common pool of progenitors, recent studies have highlighted the compartmentalization of the VZ into molecularly distinct subpopulations, each of which giving rise to a distinct cell type (Lin and Cepko, 1999, Leto et al., 2006, Morales and Hatten, 2006, Zordan et al., 2008, Leto et al., 2012). For example, VZ progenitors express Neurogenin1 , Neurogenin2 and Ascl1. Discrete microdomains express Neurogenin1 and Neurogenin2 transiently between E10.5-13.5 while Ascl1 is expressed throughout the VZ for a longer period (Maricich and Herrup, 1999, Zordan et al., 2008, Leto et al., 2012). It seems that Neurogenin1 and Neurogenin2 are specifically important for the generation of projection neurons, although they have been shown to be expressed in some interneuron populations (Kim et al., 2011, Leto et al., 2012). In contrast, Ascl1 seems to be important for the generation of all VZ derivatives (Sudarov et al., 2011). Furthermore, expression of Pax2 is important for the differentiation of interneuronal populations

but not for glia (Maricich and Herrup, 1999). These are only a couple of examples of the multitude of factors involved in establishing microdomains within the VZ progenitor field and the subsequent production of multiple cell types. Clearly, the process of generating multiple cell types from this progenitor pool is complex and dynamic.

1.6.1. Purkinje Cells

The earliest born cells from the VZ are PCs from specified neural precursors. During cerebellar maturation, PCs must be generated, migrate to their target layer and develop molecularly discrete subpopulations of PCs (Ozol et al., 1999, Marzban et al., 2010). At E11.5-12.5 in the mouse, PC progenitors express Lhx1 and Lhx5 and are exiting the cell cycle and migrating away from the VZ on the long axon fibers of RC2-expressing radial glia (Morales and Hatten, 2006, Zhao et al., 2007). The expression of Lhx1 continues in the developing PCL through E18.5, while Lhx5 is down-regulated beginning around E14.5. In addition, at E14.5 Calbindin begins being expressed in PC progenitors (Wassef et al., 1985, Zhao et al., 2007). Lhx1 and Lhx5 double mutant mice analyzed at E18.5 had small cerebella, immature folia, no PCL and only few Calbindin-positive cells. Most of these animals died shortly after birth. The Lhx family is an important factor for differentiation in many cell types (Kania et al., 2000, Shirasaki and Pfaff, 2002, Zhao et al., 2007). Furthermore, these double mutant animals still had Pax2-expression, suggesting proper development of inhibitory interneurons from the VZ, identifying Lhx1 and Lhx5 as important differentiation factors specifically in PC development (Zhao et al., 2007). It is clear that Lhx factors are important in the differentiation of PCs from the VZ.

Once PCs are generated they must survive, mature and form a fully developed and organized PCL. PC survival is dependent on tightly regulated concentrations of BDNF and NT-4 (Larkfors et al., 1996, Morrison and Mason, 1998). In addition, PC survival and differentiation are markedly improved when PCs are co-cultured with GCs, their normal in vivo pre-synaptic contacts (Baptista et al., 1994). Beyond survival, PCs have a complex process of maturation and organization into parasagittal stripes typical of adult cerebella (Consalez and Hawkes, 2012). These stripes are identical on either side of the midline and contain over ten subtypes of PCs with distinct molecular characteristics (Hawkes, 1997, Herrup and Kuemerle, 1997, Larouche et al., 2006, Apps and Hawkes, 2009). Studies have shown that these molecular expression patterns

are correlated with functionally distinct neural circuits (Chockkan and Hawkes, 1994, Hawkes and Mascher, 1994, Chen et al., 1996, Hallem et al., 1999, Apps and Garwicz, 2005, Wadiche and Jahr, 2005, Apps and Hawkes, 2009, Paukert et al., 2010, Consalez and Hawkes, 2012, Ebner et al., 2012, Graham and Wylie, 2012). It is thought that even during early neurogenesis, PCs are already divided into these functionally distinct populations. For example, cells born around E10-11.5 are destined to become Zebrin-positive while those born around E11.5-13 are Zebrin-negative later in development (Hashimoto and Mikoshiba, 2003, Larouche et al., 2006, Namba et al., 2011). By E18 the developing PCs disperse and form a single cell layer and can be recognized as molecularly distinct clusters (Herrup and Kuemerle, 1997, Marzban et al., 2007, Consalez and Hawkes, 2012). Furthermore, discrete afferent terminal domains are essential for patterning these functional units (Akintunde and Eisenman, 1994, Ji and Hawkes, 1994, 1995). For example, climbing and mossy fiber terminal fields coincide with these parasagittal stripes of PCs (Ji and Hawkes, 1994, Sillitoe et al., 2003, Voogd and Ruigrok, 2004, Sugihara and Quy, 2007, Armstrong et al., 2009). Finally, the most rapid maturation of PCs occurs between P12-18, with drastic changes in the electrophysiological and morphological properties of these cells (McKay and Turner, 2005). It is clear that multiple factors and mechanisms are essential for generating a fully mature PCL from VZ progenitors.

1.6.2. Interneurons and Glia

In addition to generating PCs, the pool of VZ progenitors includes those that will give rise to interneurons and glia. After extensive *Shh*-mediated proliferation, large clusters of progenitors delaminate and migrate into the developing white matter where they mature (Zhang and Goldman, 1996a, b, Milosevic and Goldman, 2002, 2004, Anthony et al., 2005, Grimaldi et al., 2009, Huang et al., 2010). Essentially, the VZ germinal zone moves into the developing white matter and these progenitors are sensitive to external cues, maintaining their flexibility in terms of proliferative capacity, cell fate and differentiation late into postnatal life (Leto et al., 2006, Consalez and Hawkes, 2012, Leto et al., 2012). Around E12, these VZ derivatives are able to give rise to any type of inhibitory interneuron or glia; however, later in development these progenitors undergo progressive fate restriction (Leto et al., 2012). Ascl1 is one factor thought to be highly important in the fate determination of interneurons over glia from a common pool of progenitors later in development. For example, studies in Ascl1 mutants and knockouts reveal

that these animals have a decrease in interneuron populations that is complimented by an increase in astrocyte populations (Grimaldi et al., 2009, Sudarov et al., 2011). In addition, Pax2 expression seems to be restricted to inhibitory interneurons beginning at E12, as these cells exit the VZ (Maricich and Herrup, 1999, Weisheit et al., 2006, Leto et al., 2009). All of this evidence highlights the importance of particular protein expression and environment on the cell fate choice of interneurons and glia from a common flexible pool of progenitors in the white matter.

These proliferating progenitors in the white matter will give rise to all of the subclasses of inhibitory interneurons of the cerebellum between E12.5 and P15, peaking around P5 (Weisheit et al., 2006, Leto et al., 2008). The array of mature interneurons that this population gives rise to can be distinguished by morphological, functional and chemical properties (Schilling et al., 2008). Interestingly, it seems that the generation of interneurons occurs in an inside out manner beginning with the DCN interneurons, followed by the GCL interneurons and finishing with the ML interneurons (Geurts et al., 2001, Laine and Axelrad, 2002, Yamanaka et al., 2004, Hoshino et al., 2005, Weisheit et al., 2006, Simat et al., 2007a, Simat et al., 2007b, Schilling et al., 2008, Lundell et al., 2009, Uusisaari and Knopfel, 2011, Consalez and Hawkes, 2012). The fate of these progenitors depends extensively on the extracellular environment, including cell-cell interactions, secreted factors and extracellular matrix cues revealing position, and cell cycle length (Leto et al., 2006, Leto et al., 2008, Schilling et al., 2008, Lee et al., 2009, Leto et al., 2010, Leto et al., 2012). Clearly multiple factors are involved in the generation of this varied group of inhibitory interneurons from a large group of flexible progenitors.

The final large cell population generated from the VZ, and from this cluster of white matter progenitors, is glia. Many glial subtypes arise from common progenitors, similar to the interneurons, making it difficult to track and identify particular glial populations. As mentioned previously, glial precursors delaminate from the VZ and migrate into the white matter just before birth, where they continue to proliferate before they finally differentiate into mature glia by the 3rd week of life (Miale and Sidman, 1961, Fujita et al., 1966, Levine et al., 1993, Miyake et al., 1995). Although the large pool of VZ progenitors gives rise to all glial populations, there seems to be distinct molecular expression patterns that differentiate each population into their respective mature cell type. Therefore, precise protein expression patterns are imperative to direct these progenitor pools to generate the variety of glial cells found in the adult cerebellum.

1.7. Rhombic Lip Derivatives

The RL is the second germinal zone of the developing cerebellum and has been the subject of an abundance of experimental research in the past few decades. It is a unique tissue that arises at the interface of the neural tube and roof plate (RP) and gives rise to excitatory cells of the cerebellum and precerebellar system. Although it is thought of as a distinct germinal region, the exact borders of the RL remain elusive. It is anatomically obvious beginning around E13 when there is a bend in the neuroepithelium and the initial formation of the EGL (Wang and Zoghbi, 2001, Wingate, 2001). Furthermore, the inductive cues and origin of the RL are only beginning to be understood. The RL originates from rhombomere 1 (Wingate, 2001) and BMP signaling from the RP is essential for its induction, as well as for the generation of its derivatives (Alder et al., 1999). Once generated, it is an interplay of molecular expression patterns that establishes distinct regions and progenitor pools in the RL.

The RL gives rise to the excitatory cell types of the cerebellum, including the DCN neurons, GCs and UBCs. From E10.5-12.5, glutamatergic DCN projection neurons are the first cerebellar neuronal population to be generated from the RL. After leaving the RL these cells migrate up and over the cerebellar primordium to the nuclear transitory zone (NTZ), before finally settling into distinct nuclei in the white matter (Machold and Fishell, 2005, Wang et al., 2005, Fink et al., 2006, Machold et al., 2007, Leto et al., 2012). Subsequently, GC progenitors migrate up this same path to form the EGL between E12-17 (Machold and Fishell, 2005, Leto et al., 2012). The final population of cells generated from the RL is the UBC pool between E14-E21. This progenitor population will give rise to glutamatergic interneurons of the GCL (Nunzi et al., 2001, Nunzi et al., 2002, Sekerkova et al., 2004, Leto et al., 2012). Much research has recently gone into attempting to understand the signaling factors involved in generating multiple distinct cell types from a single germinal region. These studies have revealed that perhaps the RL is, in fact, parsed into distinct regions (Chizhikov et al., 2010, Yeung et al., 2014).

Recent studies suggest that the RL can be divided into four molecularly and functionally distinct domains. One compartment is comprised of cells in the RP and distal RL that expresses *Wls* and low levels of *Math1* and *Lmx1a* from E11-18. The interior cells of the RL express *Wls* and form a second compartment at E13.5 that does not express Pax6 or *Lmx1a* and co-labels minimally with *Math1*. A third compartment is in the exterior RL and forming EGL that strongly expresses *Math1* and Pax6 and is likely a derivative from the interior *Wls*-expressing region.

There is a final compartment of cells appearing around E15.5 between the interior and exterior regions of the RL that expresses *Tbr2*, *Lmx1a* and Pax6, but not *Wls*. This final compartment of cells does not seem to appear from the *Wls*-expressing region, suggesting a novel progenitor domain for this cell population (Chizhikov et al., 2010, Yeung et al., 2014). Furthermore, it has been suggested that these domains may be functionally distinct. For example, cells in the external RL have a much higher proliferative capacity compared with the cells in the interior RL (Yeung et al., 2014). Furthermore, DCN neurons arise between E10.5-12.5 from the *Math1*-expressing region and not from a lineage of Lmx1a- or Wls-expressing cells (Machold and Fishell, 2005, Chizhikov et al., 2010, Yeung et al., 2014). In addition, GCs born around E12.5 strongly express *Math1* and Pax6, but not *Lmx1a*, suggesting that they arise from the external RL domain that shares this expression pattern (Engelkamp et al., 1999, Machold and Fishell, 2005, Chizhikov et al., 2010, Yeung et al., 2014). The final cell type that arises from the RL are the UBCs and they seem to originate from the latest developing domain that expresses *Tbr2*, *Lmx1a* and Pax6, but not *Wls* (Chizhikov et al., 2010, Yeung et al., 2014). These results suggest that overlapping regions characterized by molecular expression can subdivide the RL into distinct domains. However, the exact functional differences between these domains and the possible lineage restriction between progenitor pools are still unclear.

1.7.1. The Role of Math1 in the Formation and Development of the Rhombic Lip

As mentioned, *Math1* is an important marker of the RL and its derivatives. *Math1* is a bHLH transcription factor that is homologous to *Drosophila* atonal (*Atoh1*) (Akazawa et al., 1995, Ben-Arie et al., 1997). It begins being expressed at around E9 in the mouse RL and is regulated by Notch1 signaling in the developing cerebellum and BMP signaling from the RP (Akazawa et al., 1995, Leto et al., 2012). In addition to giving rise to the known cerebellar derivatives, including both DCN neurons and GCs, the *Math1*-expressing RL also gives rise to cells of the basilar pontine nucleus, vestibular nucleus, lateral lemniscus and pontomesencephalic tegmentum, among other regions involved in the proprioceptive system (Bermingham et al., 2001, Machold and Fishell, 2005, Wang et al., 2005, Morales and Hatten, 2006). Further evidence for the importance of *Math1* has been elucidated by analyses performed on *Math1* null mutant mice. These mice completely lack an EGL, NTZ and have an underdeveloped RL (Ben-Arie et al., 2000, Wang et al., 2005, Yeung et al., 2014). Although *Math1* is expressed

throughout embryonic development in the RL, its expression in progenitors at different times seems to coincide with distinct cell types. For example, when inducible Math1Cre animals were dosed with Tamoxifen at E10.5 or E11.5, labeled cells were later found in the NTZ or DCN or in nuclei of the anterior hindbrain. However, this dosing time point did not seem to label GCs at this time. When animals were dosed at E12.5 *Math1* expression was restricted to GCs. It is clear that *Math1* is expressed in all cerebellar cell types at different times as labeling at different time points predominantly labeled distinct cell populations. These results suggest, as well, that each progenitor population is renewed and does not arise from the progenitor domain of the previously generated cell type. For example, if *Math1* is expressed in DCN neurons and their progenitors at E11.5 and these progenitors subsequently gave rise to GCs then you would expect both cell types to be labeled with an early dose time point. However, this was not the case, suggesting that the RL regenerates novel *Math1*-positive progenitor pools for each distinct cell population (Machold and Fishell, 2005). Clearly *Math1* plays an important role in the generation of each cell type from the RL and complex signaling mechanisms are involved in regulating its discrete temporal expression.

Recent studies have also highlighted the potential role of *Math1* in regulating other important developmental factors and in renewing progenitor populations in the RL. Results revealing the quick turnover of GC precursors highlights the need for a reservoir pool of progenitors to "fill in the RL" once GC precursors move into the EGL right after they are generated. Interestingly, the canonical WNT effector protein β-catenin can activate expression of *Math1* in cultured neural progenitor cells (Shi et al., 2010). Since β-catenin acts downstream of *Wls*, it is possible that *Wls* may induce *Math1* expression in the RL (Bartscherer et al., 2006, Fu et al., 2009). In addition, *Wls* mRNA and protein is highly expressed between E12.5-18.5 in the RL and CP (Yeung et al., 2014). Interestingly, throughout development, *Wls* is restricted to expression in the interior RL and is not expressed in cells of the exterior RL. These results suggest that *Wls*-expressing cells may, in fact, serve as the reservoir, inducing *Math1* expression in cells as they move into the external RL to become GC progenitors (Yeung et al., 2014).

In addition, Pax6 has been shown to be a necessary transcription factor for proper GC development, hypothesized to regulate, and be regulated by, *Math1* (Engelkamp et al., 1999, Swanson et al., 2005). Furthermore, Pax6 null mutant animals have an up-regulation of *Wls* and an expansion of the interior RL domain into the immature EGL (Ha et al., 2012, Yeung et al.,

2014). It is hypothesized that Pax6 negatively regulates *Wls* expression to regulate the downstream targets, such as *Math1* (Yeung et al., 2014). Furthermore, *Math1* null mice lack Pax6 expression, suggesting that Pax6 is positively regulated by *Math1* (Wang et al., 2005, Englund et al., 2006, Fink et al., 2006). In addition, it is clear that a tight regulation of *Math1* expression is essential for proper GC development, highlighting the need for a system to ensure this (Helms et al., 2001). These results suggest a possible mechanism by which *Math1* regulates its own expression to regulate and renew progenitor domains; however, all of the mechanisms involved are still not understood.

The last decade has elucidated a lot of knowledge concerning the development and differentiation of RL derivatives. For example, it is now known that the RL may, in fact, actually be divided into functionally and molecularly distinct domains. However, more research is needed to better understand the relationship between these domains, as well as between the progenitor pools of each cell type. Therefore, more studies aimed at looking for new cell type markers and for factors involved in regulating distinct phases of cell type neurogenesis are required.

1.7.2. Cerebellar Nuclei Neurons

The first-born cerebellar cells from the RL are the excitatory projection neurons of the DCN. Rodents have three nuclei: the fastigial, the posterior interposed and the dentate nucleus (Voogd, 2004). These neurons are born between E10.5-12.5 from *Math1*-expressing progenitors in the RL (Machold and Fishell, 2005). Once generated in the RL, these cells migrate rostrally in the EGL to the NTZ between E12.5-14.5. The NTZ is important for the maturation of DCN. Cells cluster in the NTZ during neurogenesis and continue to differentiate before they finally migrate ventrally to their final destination in distinct nuclei between E14.5-16.5 (Altman and Bayer, 1985). The NTZ is also important for the integration of the glutamatergic projection neurons and inhibitory interneurons that will both contribute to the DCN. For example, the *Ptf1a*-expressing GABAergic interneurons migrate from the VZ up into the NTZ to cluster with the *Math1*-expressing RL-derived radially migrating projection neurons to establish condensed nuclei (Hoshino et al., 2005, Wang et al., 2005, Fink et al., 2006). During development, a handful of markers are known to identify DCN neurons. For example, at E13.5, *Lmx1a* is expressed in the precursors of the DCN neurons that have migrated to the NTZ (Chizhikov et al., 2010). In addition, *Tbr1* is expressed in the fastigial nucleus of the mouse and clearly demarcates

this cluster of cells at E16.5 (Fink et al., 2006). In contrast, the dentate nucleus of the mouse expresses *Lhx9* (Wilson et al., 2008, Green and Wingate, 2014). Overall, DCN neurons are generated from the RL and migrate over the developing anlage to inhabit the NTZ before finally settling deeper to form distinct DCN.

1.7.3. Unipolar Brush Cells

The final cell type that originates from the RL are the UBCs, a population of glutamatergic interneurons within the GCL (Englund et al., 2006, Consalez and Hawkes, 2012). These cells are born from the RL around E15-P2 in the mouse (Abbott and Jacobowitz, 1995, Sekerkova et al., 2004, Englund et al., 2006, Chung et al., 2009). After being born in the RL, these cells can follow two distinct migratory routes. They can either migrate through the developing white matter to fill in the GCL or they can move up into the EGL and migrate into the GCL from there (Abbott and Jacobowitz, 1995, Chung et al., 2009). In addition, some of these cells will migrate rostrally, parallel to the VZ, traveling further into the developing brainstem (Englund et al., 2006). Once mature in the GCL, UBCs receive mossy fiber inputs and synapse on surrounding GCs (Dino et al., 2000, Dino et al., 2001). Although these cells share a layer with the most abundant cell type in the CNS, and are likely very important, they have not been the subject of a large number of studies. More research is needed to better understand the development of this population and the distinction of these progenitors from other RL derivatives.

1.7.4. Granule Cells

GCs are the second-born cerebellar cell type from the RL. Although they will mature into only a single layer of the cerebellar cortex, they are the most abundant cell type in the CNS. Due to their need for a long proliferative phase, and their two-phase migration, this population has been extensively studied to answer questions concerning the mechanisms controlling proliferation, differentiation and migration (Hatten and Heintz, 1995, Dahmane and Ruiz i Altaba, 1999, Karam et al., 2000). After GC precursors are born in the RL they proliferate and mature in the EGL, a transient layer on the surface of the developing cerebellum (Hallonet et al., 1990). After exiting the cell cycle and beginning to mature, GC progenitors migrate inward to

establish the GCL (Logan et al., 2002). Many studies have attempted to understand the mechanisms involved in each step of this phase of neurogenesis.

GCs begin being generated around E12.5 from the *Math1*-expressing RL. Fate-labeling studies have revealed that GC precursors begin migrating into the EGL as soon as they are born. The first-born cells will mature into cells of the anterior lobes while cells born later will fill in the posterior lobes. For example, if Math1Cre animals are dosed with Tamoxifen at E15.5 it will label cells within all of the folia, while labeling at E12.5 labels only cells of the anterior folia and E17.5 dosing labels only the posterior folia (Machold and Fishell, 2005). *Math1* is expressed in the progenitors as they are born in the RL, as they migrate up into the EGL, as they continue to proliferate and is finally down-regulated when these cells migrate into the GCL (Hatten and Heintz, 1995). It has been shown that a tight regulation of *Math1* expression in these cells is imperative for their proper differentiation (Akazawa et al., 1995, Helms et al., 2001). In addition to *Math1*, these precursor cells exiting the RL express Pax6 (Stoykova and Gruss, 1994). Pax6 is also required for the proper development of GCs. For example, mice that lack Pax6 expression have a severely perturbed EGL and immature folia (Engelkamp et al., 1999, Swanson and Goldowitz, 2011). As previously mentioned, Pax6 is thought to be regulated by *Math1*, highlighting the importance of the co-expression of these two factors in the proper generation of GC precursors and mature GCs.

Once born, these precursors must migrate successfully into the EGL in order to continue to the next phase of neurogenesis. This cohort of cells migrates rostrally in a stream that climbs up and over the developing cerebellum, often crossing the midline (Hallonet et al., 1990, Lin and Cepko, 1998, Lin et al., 2001). As mentioned previously, the first-born cells are the ones that will inhabit the anterior folia, meaning that these cells will migrate a longer distance, filling in the rostral EGL, while later born neurons will migrate a shorter distance (Wingate and Hatten, 1999, Machold and Fishell, 2005). Migration begins parallel to the midline in the rostral direction from their parent cell in the RL. The cells in this phase of migration are polyhedral and reside in the superficial layer of the EGL. Once this phase of migration is complete, cells become unipolar and move into the inner region of the outer EGL. The cells in this inner region of the EGL are able to migrate both mediolaterally and raustrocaudally. Both of these migratory zones are still more superficial than the post-proliferative inner EGL (Ryder and Cepko, 1994, Wingate and Hatten, 1999). These phases of migration do not have discrete time points and are overlapping,

such that some cells finish proliferation and undergo mediolateral migration, while other cells are still in the initial phase of rostrocaudal migration (Ryder and Cepko, 1994). Successful migration of cells up into the EGL is essential for proper development.

Once these precursors have reached their temporary destination in the EGL they begin their proliferation phase. These cells can be identified by their expression of Pax6, *Math1*, *Zic1*, *Zic3*, and Meis1 (Lin et al., 2001). In the mouse, it is between E17 and P14 that there is the most extensive proliferation of precursors in the EGL (Smeyne et al., 1995, Dahmane and Ruiz i Altaba, 1999, Wallace, 1999, Lewis et al., 2004). GC precursors are sensitive to *Shh* signaling during this proliferative phase (Wechsler-Reya and Scott, 1999). For example, if EGL transplants from E11 (HH stage 37) chicks are treated with *Shh*, they up-regulate expression of *Gli1* and *Zic1*, both of which are transcriptional targets of *Shh* signaling in GCs. In addition, an increase in *Shh* signaling also results in increased mitosis, neurite outgrowth and migration of GCs. In contrast, if E5 chicks (HH stage 25-28) are given anti-*Shh* hybridoma cells, there is an extreme decrease in proliferation of GC precursors. Furthermore, when P1-3 mouse EGL explants are treated with anti-*Shh* there is a 2-fold decrease in proliferation (Dahmane and Ruiz i Altaba, 1999). Surprisingly, GCs do not express *Shh* throughout most of development; however, PCs do secrete *Shh*. For example, PC-secreted *Shh* can result in an up-regulation of *Gli1* in surrounding cells and subsequent induction of cyclin D1 and MycN (Hatten and Roussel, 2011, Roussel and Hatten, 2011). Furthermore, *Ptf1a*-null animals that do not have PCs have an increase of apoptosis in the EGL, possibly resulting from a lack of *Shh* secretion from PCs, highlighting the importance of this signaling in the survival and differentiation of GC precursors (Dahmane and Ruiz i Altaba, 1999, Lewis et al., 2004, Hoshino et al., 2005). All of these results highlight the important role of *Shh* signaling in the proliferation of GCs in the EGL.

The next phase of neurogenesis of GCs is the inward migration and differentiation of cells from the EGL to the GCL. GC precursors migrate along the processes of Bergmann glia through the developing PCL, and undergo differentiation as they migrate (Roussel and Hatten, 2011, Buffo and Rossi, 2013). In addition, it is thought that BDNF from PCs induces the mass inward migration of immature GCs (Borghesani et al., 2002). It is around E8 (HH stage 33-35) in the chick that post-mitotic bipolar GC precursors begin migrating inward to form an inner layer in the EGL (Logan et al., 2002). Subsequently, these cells continue to migrate further inward to inhabit the GCL after E9 (HH stage 35), in the chick (Karam et al., 2000, Mecha et al., 2010).

This inward migration occurs in raphes, or "ribbons", established by differential molecularly expression (Karam et al., 2000, Consalez and Hawkes, 2012). For example, as cells migrate inward they move through segments with differential *EphA4*, *EphB2*, *EphA3* Ephrin-B1 expression (Karam et al., 2000) Migration in these stripes is also dependent on the striped expression of Cad7 in migrating cells (Arndt et al., 1998). As cells migrate they express NeuroD1, *Zic1* and Pax6 (Miyata et al., 1999, Lin et al., 2001, Mecha et al., 2010). The expression of NeuroD1 during this process is imperative as it is thought to be essential in down-regulating the expression of *Math1*, and thus in terminating the proliferative phase and promoting maturation and migration. For example, if NeuroD1 is expressed ectopically in the RL of chick embryos at E4 (HH stage 22-25), the proliferation in the EGL is decreased, followed by a reduction in migration into the GCL (Butts et al., 2014). In addition to NeuroD1, migrating and differentiating GCs express NeuN, a marker of mature neurons (Mecha et al., 2010). There is a peak of migration around E16 (HH stage 42) in the chick, after which time the ribbons disappear (Feirabend, 1990). This phase of migration and differentiation from the proliferative EGL to the post-mitotic mature GCL is dependent on complex signaling and is a sensitive and dynamic process.

As cells fill in the GCL, they have historically been looked at as a homogenous population; however, more recent evidence suggests that GCs can actually be divided into distinct subpopulations (Consalez and Hawkes, 2012). For example, GCs have differential expression of *Otx1*/2, NADPH-oxidase, *Fgf1*, Eph receptors and Ephrins (Frantz et al., 1994, Hawkes and Turner, 1994, Alam et al., 1996, Ozol and Hawkes, 1997, Rogers et al., 1999). Since GCs migrate through clusters of PCs, it is possible that they acquire this patterned organization as a result of differential molecular and environmental cues as they undergo differentiation. This hypothesis would mean that GC clusters mature into cells that are functionally similar to the PCs through which they migrate, and in fact, compartmentalization between the two cell types seems to coincide. However, this is not known for sure, and in turn it is possible that GC differentiation is more dependent on the input from the mossy fibers (Karam et al., 2000, Luckner et al., 2001, Consalez and Hawkes, 2012). Specifically, it is not known whether these distinct GC clusters result from discrete lineages and increased fate restriction, from intrinsic processes, or whether it is epigenetic regulation as a result of environmental cues from PCs or mossy fibers (Consalez and Hawkes, 2012). The idea of lineage restriction in RL GC progenitors has been of interest

lately, since early born GCs around E12.5-15.5 inhabit anterior lobes while later born cells fill in posterior lobes, and these coincide with functional differences (Machold and Fishell, 2005).

1.8. Cut/Cux Orthologs and Homologs

The Cut/Cux family of proteins was first identified in *Drosophila melanogaster.* The Cut locus is large and complex. For example, depending on the location of the mutation, the resulting phenotypes can be dramatically different (Johnson and Judd, 1979, Blochlinger et al., 1988, Liu et al., 1991). The protein has a molecular mass of 240kD and contains homeodomains near the carboxyl terminus. These domains are 61 amino acids long and allow the protein to bind to specific DNA sequences due to the general homeodomain helix-turn-helix structure that is able to fit into the large groove of DNA (Quaggin et al., 1996). Homeotic genes usually differ greatly in primary sequence outside of the homeoboxes and this family of genes direct a variety of developmental processes (Scott et al., 1989). The Cut homeodomain, although somewhat different from other members of the homeobox-containing family, retains the nine amino acids characteristic of all previously identified homeodomains. Other regions of the homeodomain differ from most other homeoproteins, suggesting that this family may have unique DNA binding properties (Quaggin et al., 1996). Furthermore, this protein is extremely important for proper development of the external sensory organs in *Drosophila*. For example, lethal mutations in the Cut gene can cause the external sensory organ domain to develop into chordotonal organs (Bodmer et al., 1987). The role of Cut was further elucidated when it was found to be a Notch effector in wing development (de Celis et al., 1996, Doherty et al., 1996, Micchelli et al., 1997). These are only a few of the numerous studies in *Drosophila* that have highlighted the very important role of this protein family in proper embryonic development.

This gene has a mouse homologue, termed Cux1, which was discovered in N2a mouse neuroblastoma cells (Valarche et al., 1993). While attempting to clone Cux1, a new cut-like protein was discovered that also resides on chromosome 5. The new protein, termed Cux2, was found to be 65 % identical to the nucleotide sequence of murine Cux1, including a homeodomain on the carboxyl terminus that is 75% identical. Cux2 is also 29% identical and 50% similar to the Cut protein in *Drosophila*. Furthermore, Cux2 also contains three 60 amino acid cut repeats that are 88-98% identical to Cux1 for all three repeats (Quaggin et al., 1996). The high degree of similarity between Cut, Cux1 and Cux2 suggests similar functions between the three genes.

Furthermore, Cux2 was found to directly bind DNA (Gingras et al., 2005). Overall, it is clear that Cux1 and Cux2 likely have similar functions in regulating gene expression during embryogenesis. However, more research is needed to better understand the role of Cux2 in the generation of neural tissues.

1.8.1. Cux2 Expression in the Mouse

The expression of Cux2 in the adult mouse is limited to the brain, with reported expression in the thalamus, limbic system, cerebral cortex, external capsule, hippocampus, claustrum and endopiriform nucleus (Quaggin et al., 1996, Iulianella et al., 2003, Nieto et al., 2004, Iulianella et al., 2008, Yamada et al., 2015). Particularly, Cux2 seems to be most highly expressed in regions involved in sensory processing, such as the ventroposterior thalamic nuclei, piriform cortex, amygdala and peripheral ganglia of the glossopharyngeal and trigeminal nerves (Quaggin et al., 1996, Nieto et al., 2004). These results are consistent with the important role of Cut in the development of the sensory system in *Drosophila* (Bodmer et al., 1987).

In addition to expression in adult mice, Cux2 is highly expressed in central and peripheral neural tissues of the developing embryo. For example, it is expressed in the trigeminal ganglion, telencephalon, olfactory epithelium, branchial arch, limb bud progress zones, RP, spinal cord, VZ, subventricular zone (SVZ), pons, dorsal root ganglia and urogenital tissues (Quaggin et al., 1996, Iulianella et al., 2003). As Cux2 is expressed in much of the developing nervous system, this suggests a role in generation or maintenance of neurons as opposed to gross patterning.

1.8.2. Cux2 in Cortical Neurogenesis

Cux2 is present in the developing and adult cerebral cortex and has been under extensive investigation in recent years. In the adult cortex, Cux2 is most strongly expressed in layers II-III (Zimmer et al., 2004, Cubelos et al., 2008, Franco et al., 2012, Guo et al., 2013, Gil-Sanz et al., 2015). Furthermore, between E12.5-16.5 there is an increase in Cux2 expression as cells move from the VZ to SVZ and rapidly divide. These cells then later mature into upper layer neurons, suggesting that Cux2 is important in the generation and fate determination of this neuronal population (Nieto et al., 2004, Zimmer et al., 2004, Cubelos et al., 2008, Franco et al., 2012). Further fate mapping studies using a *Cux2-Cre* driver revealed that 76% of *Cux2*-expressing progenitor cells matured into layer II-IV cortical neurons. These results were confirmed with

culture experiments in which 80% of *Cux2*-positive progenitors became mature neurons with characteristics of upper layer neurons. Furthermore, when *Cux2* was over-expressed by *in utero* electroporation at E12.5 and analyzed at P10, 83% of the *Cux2* electroporated cells were found in layers II-IV of the cortex, further supporting the hypothesis that *Cux2* is important for fate restriction of radial glial progenitors to upper layer neurons (Franco et al., 2012). All of these results suggest the fate-restriction of *Cux2*-positive progenitors in the telencephalon and identify it as an important regulator of neurogenesis.

However, these results have become much more complicated as recent studies using similar transgenic mouse lines suggest that *Cux2*-expressing progenitors are, in fact, not fate restricted during corticogenesis. Using the inducible mouse line dosed at E10.5, it was found that P0 animals had labeled cells in all cortical layers. These results suggest that *Cux2* is expressed in multipotent radial glia progenitors that can give rise to cortical neurons of layers II-VI during development (Guo et al., 2013, Eckler et al., 2015). These results highlight the important debate concerning the fate restriction of *Cux2* during cortical neurogenesis and the potentially very complex role of this factor. In addition, it highlights the issues that can arise from using transgenic animals. Therefore, during corticoneurogenesis the precise role or fate restriction of *Cux2* is still unclear.

1.8.3. Cux2 in the Olfactory Epithelium

Cux2 is also highly expressed in the olfactory epithelium during neurogenesis. Surprisingly, it is never expressed in mitotic cells, but rather only in cells that have left the cell cycle. Experiments in the olfactory epithelium have helped elucidate the role of Cux2 in relation to other developmental signaling pathways, such as Notch signaling. In this region Cux2 still seems to function in the Notch signaling pathway, although differently than in other neuronal regions and to *Drosophila* (de Celis et al., 1996, Doherty et al., 1996, Micchelli et al., 1997). For example, when *Cux2* is overexpressed directly in the developing olfactory epithelium of chick embryos it resulted in an inhibition of Notch signaling, proliferation and neuronal differentiation. In addition, when *Cux2*-siRNA was electroporated into chick embryos they had a complete absence of progenitor cells, neuronal precursor cells, post-mitotic neurons, mitotic neurons and Notch-ligand expressing cells (Wittmann et al., 2014). Furthermore, by E13.5 $Cux2^{-/-}$ mouse embryos showed a reduction in progenitor cells, mitotic cells, neuronal precursors, post-mitotic

neurons and Notch expression in the olfactory epithelium. These results suggest that in the mouse and chick, *Cux2* is essential for maintaining the pool of proliferating cells during neurogenesis and that too much or too little *Cux2* results in decreased neurogenesis. These experiments were able to successfully reveal that Cux2 functions in the Notch signaling pathway and is able to regulate proliferation and differentiation; however, the mechanism by which *Cux2* regulates proliferation is still not completely understood.

1.8.4. Cux2 in the Hippocampus

Cux2 is also highly expressed in the hippocampus, specifically in the dentate gyrus (Quaggin et al., 1996, Yamada et al., 2015). Strong Cux2 expression begins at E14.5 in the ventricular region of the medial telencephalon, the site of dentate gyrus formation. At E16.5, Cux2-expression in the SVZ is restricted to cells that will later become granule cells. In addition, Cux2 is strongly expressed in Calretinin-positive newborn granule cells migrating out of the subgranular zone. This expression pattern was confirmed using the *Cux2-ires-Cre;R26r-tdTomato* to fate map the hippocampus, which showed restricted expression during development in the subgranular zone and a thin layer in the outer region of the granule cell layer. By adulthood, almost all granule cells in the granule cell layer were tomato-labeled. Overall, it is clear from these experiments that in the hippocampus, *Cux2* is uniquely expressed in granule cell progenitors (Yamada et al., 2015). Interestingly, Cux2 seemed only to be expressed in non-proliferative progenitor cells, suggesting a restricted expression to post-mitotic cells in this region, in agreement with the expression in the DRG and olfactory epithelium (Wittmann et al., 2014). Overall, Cux2 seems to identify a pool of non-self-renewing subgranular zone progenitors that will give rise to granule cells of the hippocampus; however, the exact role in the generation of these cells remains elusive (Yamada et al., 2015).

1.8.5. Cux2 in Spinal Cord Neurogenesis

As mentioned previously, Cux2 is expressed in the spinal cord during embryogenesis and seems to be important for proliferation in this region. Cux2 expression is initiated at E9.5 and by E10.5-E11.5 it is highly expressed in Pax6-positive proliferating neuronal precursors in the VZ, developing and post-mitotic ventral interneurons of the marginal zone, new neurons that are exiting mitosis in the intermediate zone, but only rarely in developing and mature motor neurons.

Specifically, Cux2 often co-labeled with $p27^{Kip1}$ in cells that were exiting the VZ, exiting the cell cycle and undergoing terminal differentiation (Iulianella et al., 2008). Further studies in *Cux2* hypomorphs revealed that Cux2 normally acts to promote terminal differentiation of ventral and dorsal interneurons and to inhibit motor neuron generation (Iulianella et al., 2008, Iulianella et al., 2009). Furthermore, E11.5 *Cux2* hypomorphs had a reduction in neural tube size, a loss of axonal mass and a decrease in progenitors and differentiated cells of the marginal zone compared to controls (Iulianella et al., 2008, Iulianella et al., 2009). Although there was a decrease in size, the overall spinal cord morphology did not seem grossly affected, suggesting that *Cux2* is not necessary for initial spinal cord patterning (Iulianella et al., 2008). Furthermore, when *Cux2* was overexpressed using a Nestin promoter, animals had a slightly larger spinal cord at E10.5-11.5, with a more dramatic increase in the marginal zone compared to the VZ (Iulianella et al., 2008). Overall, these results again highlight *Cux2* as an important factor in regulating proliferation and differentiation of interneurons, specifically of progenitors in the marginal zone, during spinal cord development.

1.8.6. Cux2 as a Cell Cycle Regulator

Many studies have elucidated the role of Cux2 in regulating the cell cycle. Cux2 is expressed in the cell cycle just before and during G2 and/or M-phase and during the last division of progenitors (Zimmer et al., 2004). This oscillation of expression at different points during the cell cycle is reminiscent of the expression pattern of Cux1, which increases during the G1/S transition and then decreases again during the G2/M transition (Coqueret et al., 1998, Moon et al., 2001, Santaguida et al., 2001). Therefore, it is not surprising that E10.5 Cux2 hypomorphs had a much larger number of spinal cord VZ cells in S phase, an increase in the length of S phase and a decreased number of cells in G2/M-phase compared with controls. In addition, Cux2 expression peaks around E11.5, and at this time these animals had a reduction in VZ mitotic events and in Neurod1-expressing post-mitotic cells, highlighting a consequential decrease in proliferation and differentiation as a result of Cux2 loss (Lee et al., 1995, Lee et al., 2000, Iulianella et al., 2008).

In contrast to the results in the spinal cord, the cortex of *Cux2* mutants were larger and displayed an increase in proliferating cells in upper cortical layers and the SVZ (Cubelos et al., 2008). Furthermore, the neural progenitor cells in the SVZ exhibited a 2.5-fold increase in the

number of precursor cells that re-entered the cell cycle after their first division. In addition, it was found that both Cux2-expressing and non-expressing cells maintained their intrinsic proliferative capacity when cultured at E13.5. This suggests that Cux2 is a significant marker of functionally distinct progenitor cells (Franco et al., 2012). Many studies suggest that the duration of the cell cycle can influence cell fate, highlighting this as a possible mechanism by which Cux2 is able to influence cell fate specification (Gao et al., 1997, Durand et al., 1998, Raff et al., 1998, Shen et al., 2006, Wilcock et al., 2007).

The role of Cux2 in CNS development appears to be region specific. For instance, in the DRG, olfactory epithelium, and hippocampus, Cux2 is only found in non-proliferating cells (Bachy et al., 2011, Wittmann et al., 2014, Yamada et al., 2015). Although there are still questions concerning the exact mechanism and function of Cux2, all of these results are consistent in highlighting its important role in regulating proliferation and differentiation.

1.8.7. Upstream and Downstream Events Regulated by Cux2

In the developing spinal cord and olfactory epithelium, Cux2 functions downstream of Notch signaling to regulate neurogenesis (Iulianella et al., 2009, Wittmann et al., 2014). Interestingly, in the olfactory epithelium Cux2 participates in a complex feedback loop by limiting the expression of the Notch ligand Delta, and potentiating the effects of BMP signaling on restricting the Notch pathway in neural precursors (Wittmann et al, 2014). Notch is able to influence progenitor differentiation in the spinal cord by regulating proneural genes (Gaiano and Fishell, 2002, Fischer and Gessler, 2007). In addition, Notch1 activity induces Cux2 expression in subsets of progenitors. Mutations in the Notch signaling pathway diminish Cux2 expression and severely perturb spinal cord development (Iulianella et al., 2009). Since Notch1 over-activation can promote the maintenance of the progenitor pool and Cux2 can act to maintain mitotic events and to promote differentiation, it is possible that Cux2 acts to strengthen developmental events, such as proliferation and terminal differentiation and is activated downstream of Notch1 signaling (Iulianella et al., 2009). For instance Cux2 binds both Neurod1 (a Notch target gene mandatory for survival and maturation of newly born cells) and $p27^{Kip1}$ promoters *in vivo* and positively regulates their expression. Consistent with this, Cux2 hypomorph embryos revealed a decrease in Neurod1-, $p27^{Kip1}$- and $p57^{Kip2}$-expressing domains in the dorsal spinal cord. These animals also had changes in the expression of other progenitor

and neuronal markers, highlighting an important role of Cux2 in progenitor pool maintenance, cell cycle withdrawal and neuronal differentiation (Gui et al., 2007, Iulianella et al., 2008, Iulianella et al., 2009). In contrast, Cux2 overexpression leads to an increase in Neurod1- and $p27^{Kip1}$-expressing cells. These results highlight the important role of Cux2 in cell cycle regulation by direct DNA binding to modulate gene expression (Iulianella et al., 2008, Iulianella et al., 2009).

Altogether, the functional studies on Cux2 highlight the important role it plays in the proliferation and differentiation of neuronal precursors. Yet there remain many puzzling observations of the exact mechanisms of how Cux factors influence neural development. For instance, it is not yet known how Cux2 can function as a cell cycle promoting factor in some regions of the developing nervous system, yet act in a strictly differentiating role in other circumstances. It highlights the complexity of the large Cut-related homeodomain subclass of proteins and demonstrates further studies are required to fully elucidate their roles in neural development.

1.9. Hypotheses

Recent studies have revealed the important role of the cerebellum in a broad array of functions, ranging from movement generation to cognition, making it an important brain region to study. In addition, developmental biology has begun elucidating the complex mechanisms controlling cerebellar development. Furthermore, GCs are the most numerous cells in the entire CNS and define an entire layer of the cerebellar cortex. Therefore, understanding the mechanisms controlling proliferation and differentiation of these cells during development has become even more important. It is well established that GCs arise from the RL, proliferate in the EGL and migrate to their final location in the GCL. In addition, it is now thought that GCs can be subdivided into functionally distinct classes. However, there are still many questions that remain concerning the mechanisms that regulate each of these processes. For example, do GCs derive from the same progenitors as other RL derivatives? Are there distinct subpopulations of progenitors or do all GCs come from a common pool? When do these cells become fate restricted? What other mechanisms control the proliferation and differentiation of all GCs and of each distinct domain?

We recently showed that Cux2 acts as a GC progenitor marker in the developing hippocampus (Yamada et al., 2015). In addition, preliminary work revealed that it was also expressed in GCs of the cerebellum. Thus, the **hypothesis** I addressed here is whether **Cux2 expression is restricted to GCs in the developing cerebellum and whether it acts as a fate determinant in this population**. Addressing this question will shed light on whether cerebellar GC progenitors are distinct from other RL progenitors by their expression of Cux2. In addition, it will elucidate whether Cux2 marks a distinct class of GC progenitors that give rise to a specific subpopulation. Finally, it will reveal whether Cux2 plays a significant role in the fate specification of these cells from RL progenitors.

My first question addressed the discrete expression pattern of Cux2 in this region throughout development. I looked at protein and mRNA expression in whole mount and sections of mouse and chick brains to characterize expression patterns. I thought Cux2 would be spatially restricted to the germinal zone of a distinct cell population. Next, I used genetic labeling strategies to fate map *Cux2*-expressing cells during development. I wanted to address the question of whether or not *Cux2* is, in fact, fate restricted during development, or whether it is involved in the generation of all cell types from a discrete location. Based on results from other brain regions, I predicted that *Cux2* would be important for the generation of a distinct cell population, as opposed to being involved in the generation of multiple cell types. Thirdly, I wanted to begin to understand the mechanism by which *Cux2* regulated the generation of a distinct cell population. In line with previous studies suggesting the importance of *Cux2* in cell cycle regulation, I overexpressed *Cux2* to determine its effect on proliferation and differentiation. My prediction was that *Cux2* overexpression would result in changes, either an increase or decrease in levels, in line with previous studies in the spinal cord and olfactory epithelium.

By analyzing the expression pattern, fate and possible mechanism of *Cux2* I was able to begin to understand its role in cerebellar development.

Chapter 2: Materials and Methods

2.1. Cux2 Protein Expression in the Developing Mouse Embryo

Timed matings of C57BL/6 mice were conducted to obtain embryos at E10.5, E11.5, E12.5, E13.5, E14.5, E15.5, E16.5 and 17.5 for a comprehensive analysis, with noon of the day of vaginal plug taken to represent day 0.5 post conception (E0.5). Pregnant dams were sacrificed by cervical dislocation and litters were harvested at the stages described above to evaluate Cux2 expression during cerebellar development. Embryos were fixed overnight in 4% paraformaldehyde (PFA)/0.1M phosphate-buffered saline (PBS) at 4˚C. The following day, they were washed 3 x 10 minutes in PBS and placed in 15% sucrose. Once equilibrated, embryos were switched into 30% sucrose. Once equilibration was complete the embryos were snap frozen in Optimum Cutting Temperature compound (Tissue-Tek, Torrance, CA) and stored at -80˚C. Embryos were sectioned parasagittally at 14µm and placed onto SuperFrost slides (VWR, Radnor, PA). Ten slides were mounted at a time to allow multiple axial levels to be captured on each slide (Appendix Ai). At least two animals were sectioned and analyzed for each embryonic stage.

Sections were permeabilized with a wash in PBS +0.5% Tween 20 (PBT) for 10 minutes and subsequently rinsed for 5 minutes in PBS. Sections were then blocked in 5% Donkey Serum in PBS for 1 hour at room temperature. The primary rabbit anti-Cux2 (Iulianella et al., 2008) antibody was diluted 1/2000 in 5% Donkey Serum in PBS and incubated overnight at 4˚C in a humidified chamber. The following morning, sections were washed 3 x 10 minutes in PBT, and incubated with a donkey anti-rabbit 488 (1:2000, Invitrogen, Carlsbad, CA) secondary antibody at room temperature for 1 hour. Sections were washed 3 x 10 minutes in PBT, counterstained with DAPI (4',6-diamidino-2-phenylindole; Sigma, St. Louis, MS) for 5 minutes, washed for 5 minutes in PBT, and mounted with Dako Fluorescent Mounting Medium. Multiple axial levels were analyzed per animal and images were captured using an Orca-flash4.0 camera using a Zeiss Observer.Z1 inverted microscope equipped with an Apotome 2 structured illumination device.

2.2. *Cux2* mRNA Expression in Developing Chick and Mouse Embryos

2.2.1. Riboprobe Preparation

Mouse and chicken-specific *Cux2* and *Shh* riboprobes were synthesized as described before (Iulianella et al., 2003, Wittmann et al., 2014). Briefly, a restriction digest was performed

at 37^{O}C to linearize *cCux2*, *mCux2*, *cShh*, and *mShh* plasmids using reagent volumes describe in Appendix Table 1. Samples were run on 1.5% agarose gel to verify that the linearization had occurred. Linearized templates were then used to make all four riboprobes by an *in vitro* transcription reaction according to the reagent volumes outlined in Appendix Table 2 and incubated at 37˚C for 2 hours. After transcription, DNase I (0.5µL) was added to the mix and incubated at 37˚C for a further 30 minutes to destroy template DNA. The reaction was terminated by the addition of 0.2M EDTA (2µL). Samples (1µL) from before and after the addition of DNase I were run on 1.5% agarose gel to confirm that the template DNA was completely degraded and riboprobe synthesis had occurred. Samples were precipitated overnight at -80˚C by the addition of 4M LiCl (2.5µL) and EtOH (75µL), spun at 14,000 rpm at 4˚C for 30 minutes, and then washed with 70% EtOH, and spun again at 14,000 rpm at 4˚C for 15 minutes. RNA pellets were then re-suspended in 25µL of pre-heated hybridization buffer (Appendix Table 3) and dissolved at 50˚C for 10 minutes. Samples were run on 1% agarose gel to confirm probe synthesis and the concentration was determined using a Nanodrop 2000 (Thermo Scientific). Samples were stored at -20˚C in hybridization buffer until needed.

2.2.2. *In Situ* Hybridization and Analysis

Isolation of chick and mouse embryos. Fertilized eggs from Dalhousie's Agricultural Campus were placed in a 37 °C humidified incubator and embryos were harvested at E2 (HH11-15), E9 (HH35-36), E10 (HH36) and E11 (HH37) (Hamburger and Hamilton, 1951). Embryos were dissected in ice cold DEPC-treated PBS. E2 embryos were separated from surrounding epithelial membranes. E9-E11 brains were removed from the heads of embryos. Mouse embryos at E13.5 were harvested and dissected in cold DEPC-treated PBS. All embryos and brains were fixed overnight in DEPC-treated 4% PFA at 4˚C. Embryos were then washed 2 x 5 minutes in DEPC-treated 0.1% PBT and dehydrated in a methanol (MeOH) series. They were consecutively washed in 25%, 50% and 75% MeOH in DEPC-treated 0.1% PBT for 5 minutes each. They were then washed 2 x 5 minutes in 100% MeOH and stored in 100% MeOH at -20 ˚C. On day 1 of the *in situ* hybridization (ISH) tissues were rehydrated in 75%, 50% and 25% MeOH in DEPC-treated 0.1% PBT consecutively for 5 minutes each. They were then washed 2 x 5 minutes in DEPC-treated 0.1% PBT to complete the rehydration process.

ISH methodology. All reagents used for this protocol were prepared using mRNA-free containers and with purchased or DEPC-treated water and PBS. All tissues were bleached with 6% peroxide in 0.1% Triton X-100 in PBS at room temperature for 1 hour. Tissues were then washed 3 x 5 minutes with 0.1% Triton X-100 in PBS. Tissues were treated with 20µg/mL Proteinase K in 0.1% Triton X-100 in PBS for 3 minutes (E2 chick embryos) or 7 minutes (E9-E11 chick brains and E13.5 mouse embryos). Tissues were washed 2 x 5 minutes in 2mg/mL Glycine in 0.1% Triton X-100 in PBS. Tissues were then washed 2 x 5 minutes in 0.1% Triton X-100 in PBS. They were then post-fixed with fresh 0.2% glutaraldehyde/4% PFA at room temperature for 20 minutes. Tissues were then washed 2 x 5 minutes in 0.1% PBT. Then 1-2mL of pre-heated hybridization buffer was added to each vial of tissues and they were incubated at 65˚C for 1 hour. This hybridization buffer was replaced with fresh pre-heated hybridization buffer containing digoxigenin-labeled chicken or mouse *Cux2* or *Shh* riboprobe and incubated overnight at 65˚C with gentle rocking. The amount of probe added varied between samples, but the total concentration was 1-2µL of probe per mL of buffer.

The following morning, tissues were washed 2 x 45 minutes at 65˚C in 2mL of 2xSSC/0.1% Chaps. Tissues were then washed for 30 minutes at 65˚C in 2mL of 0.2xSSC/0.1% Chaps. Tissues were transferred to a scintillation vile and washed for 10 minutes in Tris Buffered saline with 0.1% Tween20 (TBST; Appendix Table 4) at room temperature and then incubated in 2-4mL of blocking solution (20% heat-inactivated goat serum in TBST, 1% Boehringer blocking buffer powder, 2mM levamisole) for 1-2 hours at 4˚C. Subsequently, anti-digoxygenin-alkaline phosphatase antibody (1:2000) was added to each vile and tissues were incubated overnight at 4˚C. The following day, tissues were washed 4-5 x 1 hour in TBST and the final wash was left overnight at 4˚C. Tissues were subsequently rinsed in TBST for 2 x 10 minutes. They were then washed 3 x 10 minutes in 1mL of fresh NTMT (Appendix Table 5) at room temperature.

Color detection. To detect embryonic *Cux2* and *Shh* mRNA expression 23.6µL of NBT (100mg/mL in DMF) and 24.5µL of BCIP (50mg/mL in DMF) were diluted in 7mL NTMT buffer (NaCl, Tris, $MgCl_2$, Tween 20). This detection solution was filtered in a 20µm filter to avoid crystallization during the reaction. Embryos were incubated at room temperature protected from light for several hours to ensure adequate color development. For chick embryos, the color reaction was terminated after 1-2 hours by fixing the embryos in 4% PFA at room temperature for an hour. For mouse embryos, the reaction was allowed to continue for 3-4 hours prior to

fixation. Tissues were then washed 3 x 10 minutes in PBS and stored in 50% glycerol in PBS at 4˚C for imaging.

2.3. Genetic Fate Mapping of Cux2+ Cells

2.3.1. Generation of Mice, Tamoxifen and Fixation

For genetic fate mapping experiments delineating *Cux2*-expressing cerebellar progenitors, $Cux2^{CreERT2/+}:Rosa26^{tdTomato/tdTomato}$ mice crossed with $Rosa26^{tdTomato/\ tdTomato}$ mice were used for this study. Litters were generated using timed matings as described above. The $Cux2^{CreERT2/+}:Rosa26^{tdTomato/tdTomato}$ is an inducible mouse that results in genetic recombination of a tdTomato fluorescent gene reporter when pregnant dams are administered Tamoxifen (Fig. 2). The line was previously characterized by Franco et al (2012) to cause genetic recombination of the red fluorescent reporter gene tdTomato in *Cux2*-expressing neural progenitors. Tamoxifen (2mg/pregnant mouse; 100µL of 20mg/mL) was administered by intraperitoneal injection to pregnant dams at E10.5, E12.5 and E17.5. These time points were chosen to capture the time periods during which the majority of cerebellar cell types are generated. For example, between E10-12, PCs are generated from the VZ and DCN neurons are generated from the RL (Miale and Sidman, 1961, Goldowitz et al., 1997, Machold and Fishell, 2005, Fink et al., 2006, Morales and Hatten, 2006). Beginning at E12.5, inhibitory interneurons and glia are generated from the VZ and GCs and UBCs are generated from the RL (Dahmane and Ruiz i Altaba, 1999, Hoshino et al., 2005, Machold and Fishell, 2005, Englund et al., 2006, Chung et al., 2009, Lundell et al., 2009, Consalez and Hawkes, 2012, Leto et al., 2012). Finally, GCs are continuing to proliferate and UBCs are migrating at late embryonic stages (Smeyne et al., 1995, Dahmane and Ruiz i Altaba, 1999, Wallace, 1999, Lewis et al., 2004, Englund et al., 2006, Chung et al., 2009). Therefore, dosing at each of these stages allowed us to capture the generation of all major cerebellar cell types. Two dams were dosed at E10.5 and harvested at E16.5, while another two were dosed at E10.5 and litters were sacrificed at P14. Two dams were dosed at E12.5 and harvest at E16.5, and another two were dosed at E12.5 and litters were sacrificed at P14. Finally, two dams were dosed at E17.5 and litters were sacrificed at P14 (Fig. 3). Pregnant dams were sacrificed by cervical dislocation in order to harvest litters at E16.5. P14 litters were anaesthetized by intraperitoneal injection of 100µL of 4:1 ketamine:xylazine and perfused with

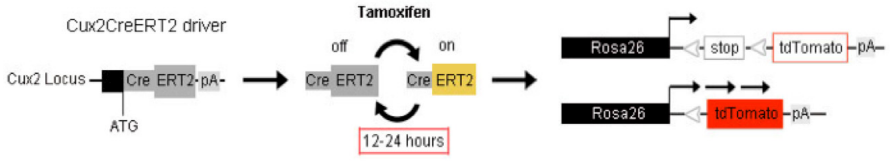

Figure 2. The genetic labeling strategy used for fate mapping of *Cux2*-expressing cells. Mice used were *Cux2*$^{CreERT2/+}$*:Rosa26*$^{tdTomato/tdTomato}$ pregnant dams. The administration of Tamoxifen causes translocation of Cre to the nucleus and subsequent recombination, resulting in the transcription of tdTomato in *Cux2*-expressing cells, allowing us to identify these cells at a later time point. Due to a 12-24 hour delay in recombination, the cells that are labeled are those that express *Cux2* 12-14 hours after the time of Tamoxifen dosing.

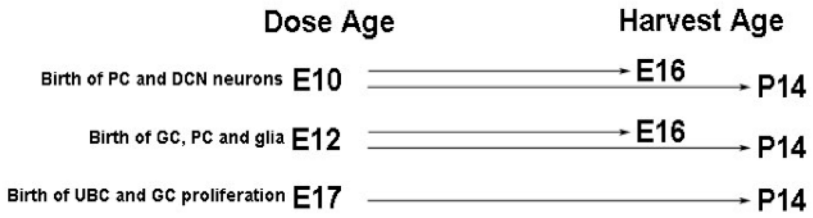

Figure 3. The dose and harvest time point combinations used for the fate mapping portion of the study. *Cux2*$^{CreERT2/+}$:*Rosa26*$^{tdTomato/tdTomato}$ mice were used and pregnant dams were dosed with 2mg of Tamoxifen to cause genetic recombination and tomato-labeling of *Cux2*-expressing cells. N=4 for each dose-harvest combination; N(total)=20.

PBS and then 4% PFA. All animals harvested at both E16.5 and P14 had brains removed from skulls. Red fluorescent brains were separated and were fixed overnight in 4% PFA at 4˚C. Brains were washed 3 x 10 minutes in PBS and equilibrated in 15% sucrose and then in 30% sucrose. Once equilibrated in 30% sucrose brains were snap frozen in Optimum Cutting Temperature and stored at -80˚C.

2.3.2. Immunohistochemistry and Analysis

Brains were sectioned parasagittally at 14µm and placed onto slides. Ten slides were mounted at a time to allow multiple axial levels to be captured on each slide (Appendix Ai). Two animals from each of two litters per time point were sectioned and analyzed ($N_{litters/stage}$=2, $N_{brains/litter}$=2, N_{total}=4 per dose-harvest combination (Fig. 2)). Primary antibodies used included rabbit anti-Calbindin (1:500, Swant, Switzerland), mouse anti-NeuN (1:500, Millipore, Temecula, CA), rabbit anti-Pax6 (1:200, Cedarlane, Burlington, ON; or 1:100, Covance, Princeton, NJ) and mouse anti-Pax6 (1:50, DSHB, University of Iowa). Secondary antibodies from Invitrogen used at 1:2000 included donkey anti-rabbit AlexaFluor 647 and donkey anti-mouse AlexaFluor 647 (Invitrogen, Burlington, ON). Images were captured from multiple axial levels from each animal to ensure analyses were being completed throughout the entire cerebellum.

Sampling methodology. Counting frames (100µm x 100µm) were placed randomly around the cerebellum on 7-20 sections per animal according to the systematic-random sampling method (Mouton, 2002, Yamada et al., 2015). The total number of cells in each box that expressed tdTomato and was co-labeled with the given marker was counted and was divided by the total number of tdTomato-expressing cells to reach an average percentage of co-labeling per animal. Percentages of co-labeling were averaged among the 4 animals per dose-harvest time point and the deviation about the mean was expressed as the variance. From this, the percentage of tdTomato-positive PCs (co-labeled with Calbindin), tdTomato-positive post-mitotic neurons (co-labeled with NeuN) and tdtomato-positive GC precursors (co-labeled with Pax6) were calculated. All images were captured using an Orca-flash 4.0 camera using a Zeiss Observer Z1 inverted microscope equipped with an Apotome 2 structured illumination device.

2.4. *Cux2* Electroporations *in ovo*

2.4.1. Electroporations and Fixation

Fertilized eggs from Dalhousie's Agricultural Campus were incubated at 37°C in a humidified chamber until E2 (HH11-15). At this stage, 3-5mL of albumin was removed from the eggs with a syringe and eggs were windowed as previously described (Krull, 2004). India ink was injected below the embryo to visualize the embryo. *Cux2-pCIG* (bi-cistronic experimental plasmid) and *pCIG* (control vector) plasmids driven by CMV and beta-actin promoters were prepared at 3.7 µg/µl in water, as described previously (Iulianella et al., 2008). The plasmid solution was injected into the developing rhombencephalon with 0.1% Fast Green solution to visualize injection. Enough plasmid was injected to fill the rhombencephalon (approximately 0.05µl or .185µg). After plasmid injection, 4-6 drops of 1x Tyrode's solution was used to moisten the embryo. ECM 830 electroporator (BTX Harvard Apparatus) was used with gold-plated electrodes to deliver five 50ms pulses at 18 volts each, with a 1 second interval between each pulse. Eggs were re-sealed with cellophane tape and placed back in the incubator until E9 (HH35-36). At this later stage, living embryos were sacrificed by decapitation and brains were removed and fixed overnight in 4% PFA at 4˚C. Brains were washed 3 x 10 minutes in PBS and equilibrated in 15% sucrose and then in 30% sucrose, snap frozen in Optimum Cutting Temperature, and stored at -80˚C until ready to section.

2.4.2. Immunohistochemistry and Analysis

Four control animals and six *Cux2*-overexpressing embryos were analyzed for this study. Electroporated brains were sectioned parasagittally at 14µm and placed onto slides (Appendix Ai). For cell fate analysis, the following primary antibodies were used: rabbit anti-Calbindin (1:500, Swant, Switzerland), mouse anti-NeuN (1:500, Millipore, Temecula, CA), mouse anti-Pax6 (1:50, DSHB, University of Iowa) and goat anti-GFP Fitc (1:500, Abcam, Cambridge, MA). Secondary antibodies used obtained from Invitrogen were diluted at 1:2000 and included donkey anti-rabbit AlexaFluro 568, donkey anti-mouse AlexaFluor 568 and donkey anti-goat AlexaFluro 488 (Invitrogen, Burlington, ON). The entire cerebellum was imaged as described above to ensure a complete analysis.

Cells in the entire cerebellar primordia were counted with a cut off line placed at the junction of the primordium and the brainstem. A total of 6-15 sections per animal were used in

the analysis. The number of GFP-positive cells co-labeled with each of the following cell-type specific markers were counted: Pax6 (GC precursors), Calbindin (PCs) and NeuN (post-mitotic neurons) were counted and normalized to the total number of GFP-positive cells in *Cux2pCIG* vs. *pCIG* electroporated controls. T-tests were performed to determine significant differences between experimental and control animals for each cell type marker (cutoff $p<0.05$).

Chapter 3: Results

3.1. Cux2 Expression in Mouse and Chick Embryos

Previous studies have shown the strong restriction of Cux2 expression in specific subsets of progenitors. For example, it is found only in progenitors that give rise to upper layer cortical neurons, in a subset of post-mitotic hippocampal granule cell progenitors, and in spinal cord interneuron populations (Nieto et al., 2004, Iulianella et al., 2008, Iulianella et al., 2009, Yamada et al., 2015). Furthermore, Cux2 has been shown to be highly important for the generation of distinct subset of neurons, highlighting it as an important factor during neurogenesis (Iulianella et al., 2008, Iulianella et al., 2009). Thus, Cux2 expression largely coincided with cell fate restriction during neural development. However, it remains unclear if Cux2 activity identifies a distinctly unipotent neural progenitor subclass in the developing nervous system. To address this we examined the role of Cux2 in the developing cerebellum, which is a simplified cortex consisting of a few well-defined cell types arising from a highly restricted region at the interface of the developing mid- and hindbrains of the vertebrate embryo. We initially aimed our study at characterizing the expression pattern of Cux2 in the developing cerebellar anlage, and then used genetic tools to investigate whether it plays a role in fate restriction within this region. By elucidating its expression and fate restriction, this study aims to reveal a possible role of Cux2 in the development of distinct subsets of cerebellar neurons.

ISH for *Cux2* and *Shh* mRNA was performed on embryonic day (E) 13.5 C57 wild type embryos (N=3). The *Shh* riboprobe was used as a control since the expression pattern during development has been well characterized in the notochord and floor plate (Ericson et al., 1995, Dodd et al., 1998). ISH for *Shh* showed clear expression in the notochord and floor plate, serving as a positive control for experimental conditions. The *Cux2* riboprobe used has previously been validated and it is specific to the 3' end encompassing all sequences from the carboxyl terminal to the third Cut repeat (Iulianella et al., 2003). *Cux2* expression was found predominantly in the developing lateral RL (Fig. 4A and 4A'), but also slightly extended around the 4th ventricle, throughout both the upper and lower RL. Interestingly, the lateral extent of *Cux2* expression was limited to the upper RL, which will contribute to the cerebellum proper. It is the germinal zone within this restricted region that will give rise to all of the excitatory neurons of the cerebellum. Moreover, this region will first give rise to DCN neurons between E10.5-12.5, then to GC

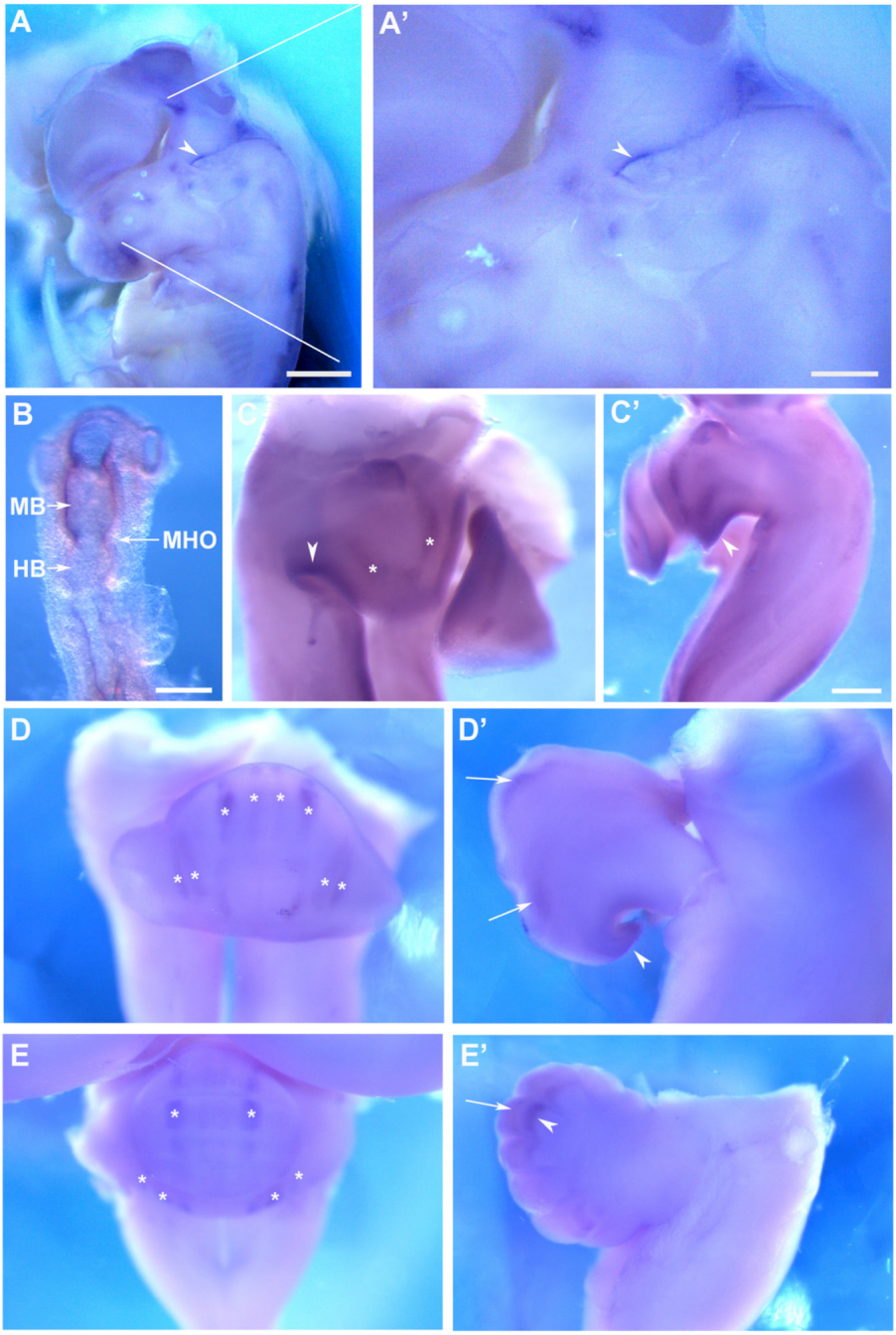

Figure 4. A series of *Cux2 in situ* hybridization experiments performed on mouse and chick embryos. E13.5 mouse embryos reveal *Cux2* expression in the developing RL (A and A'; arrow head; N=3). *Cux2* expression in the chick at E2 is found in the developing midbrain, caudal portion of the forebrain and rostral portion of the hindbrain (B; N=4). The E9 chick expresses *Cux2* in the developing RL (arrow head) and on the dorsal portion of the developing cerebellum in 2 distinct stripes (C and C'; asterisks; N=4). *Cux2* expression in the chick at E10 is found on the dorsal portion of the developing cerebellum in a clear striped pattern (D asterisks; N=2). The lateral view reveals *Cux2* expression in the RL and in the most rostral and caudal portions of the EGL (D'; arrow head and arrows, respectively). By E11 in the chick, *Cux2* is strongly expressed in stripes on the dorsal cerebellum (E; asterisks; N=2). The lateral view at this time reveals *Cux2* expression in the EGL and GCL (E'; arrow and arrow head, respectively). MB= Midbrain; HB= Hindbrain; MHO= Midbrain-Hindbrain Organizer; EGL= External Granule Layer; GCL= Granule Cell Layer. Scale bars= 1mm.

precursors beginning at E12.5 and finally to UBCs beginning at E15 (Machold and Fishell, 2005, Leto et al., 2012). In contrast, all inhibitory neurons and glia derive from the VZ, but not the RL (Dahmane and Ruiz i Altaba, 1999, Hoshino et al., 2005, Lundell et al., 2009, Consalez and Hawkes, 2012, Leto et al., 2012). Therefore, the restriction of *Cux2* to the RL at E13.5 suggests it is involved in the development of excitatory neurons.

Many of the mechanisms controlling cerebellar development are conserved between avians and mammals (Butts et al., 2014, Green and Wingate, 2014). Therefore, we wanted to determine whether the expression pattern of *Cux2* observed in the mouse was conserved in the chicken. In addition, a later portion of this study involving overexpression was performed in the developing chick, making it essential to characterize *Cux2* expression in the chick in order to validate the use of this embryonic nervous system for overexpression studies of *Cux2* in the cerebellum. ISH was performed in the chick at E2, E9, E10 and E11 (HH stages 11-13, 35, 36 and 37, respectively). The chicken *Cux2* riboprobe used has previously been validated and was prepared as described (Wittmann et al., 2014). At E2, *Cux2* mRNA was expressed along the inside of the developing neural tube, with expression being strongest in the midbrain and at the border of the midbrain-hindbrain and midbrain-forebrain (Fig. 4B). By E9, *Cux2* was strongly expressed in the RL and in 2 perpendicular lines near the medial and caudal borders of the developing cerebellum on both hemispheres (Fig. 4C and 4C'). These stripes seemed to be on the surface of the cerebellum and are reminiscent of ISH results from *Zic1*, *BMP7*, *En2* and other genes delineating PC domains (Lin and Cepko, 1998). Therefore, it is difficult to say what population of cells was expressing *Cux2* in this case; however, the expression in the RL suggests that it was GCs. The following day, at E10, the horizontal stripe at the caudal border disappeared; however, the vertical lines on either side of the midline were still obvious and 2 more lines developed lateral to each of these lines, 1 in each hemisphere (Fig. 4D). The midline stripes did not seem to have homogeneous expression in the rostrocaudal direction, with highest expression at the most rostral and caudal points (Fig. 4D asterisks). The lateral view of the E10 embryo revealed that the striped expression was, in fact, at the level of the EGL and *Cux2* was still expressed in the RL (Fig. 4D'). This striped pattern was even more obvious the following day, at E11 (Fig. 4E). The differential expression of *Cux2* within the stripes was even more dramatic at this time as well, with distinct regions within the stripes that were *Cux2*-negative. The *Cux2*-positive domains seemed to coincide with the tops of developing folia, with negative

regions lining up with the invaginations between folia. The lateral view at E11 showed *Cux2* expression still in the EGL and in a thinner deeper layer (Fig. 4E'). This deeper layer was anatomically similar to the developing GCL, which is comprised of small cells bodies and is lighter and sits ventral to the EGL (Martinez et al., 2013). *Cux2* still seemed to be highest at 2 distinct points, one in the 2 rostral-most folia and one in the caudal-most folium.

Immunohistochemistry was performed on C57BL/6 wild type embryos at each embryonic day from E10-17 (N=2-4 at each stage). Since *Cux2* mRNA clearly seemed restricted to expression in regions associated with GC development, we wanted to determine whether this pattern of expression was conserved for the protein. Furthermore, previous studies have suggested that protein and mRNA are not always expressed in the same subset of cells, prompting us to explore this possibility in the developing cerebellum (Gingras et al., 2005, Guo et al., 2013, Gil-Sanz et al., 2015). In addition, we wanted to determine whether protein expression would follow the development of GCs, in both the RL and the EGL (Machold and Fishell, 2005, Leto et al., 2012). The earliest time point we looked at was E10, and at this time we did not detect any Cux2 protein expression in the developing dorsal hindbrain at any mediolateral level (Fig. 5A). At E11.5, Cux2 protein expression was detected throughout the developing CP, with highest expression in a cluster in the middle of the invaginating tissue (arrow, Fig. 5B). The following day, E12.5, Cux2 protein expression was clear in the invaginating CP and was mainly expressed in clustered cells in the middle of the developing CP (arrow, Fig. 5C, M). In contrast, IHC using a Cux1 antibody revealed that Cux1 was expressed in the entire developing CP, RP and RL and into the cerebellar primordium. By the next day, Cux2 protein was still expressed in clustered cells in the middle of the CP (Fig. 5D). These results were striking since at this same stage (E13.5), *Cux2* transcripts were much more broadly expressed in the developing RL, and not only the CP primordium (Fig. 4A-C'). From E14.5 to E15.5, Cux2 protein decreased slightly, as it did not seem to be as widespread in cells of the developing CP, and it was still not expressed in the developing cerebellum (Fig. 5E, 5F). Cux2-expressing cells were most often observed in the middle of the developing CP, often being sandwiched by Cux2-negative regions of CP tissue (arrow, Fig. 5F). There was some variability in Cux2 expression in the mediolateral direction, with some sections having Cux2 expression throughout the developing CP and some sections having only a handful of Cux2-positive cells in

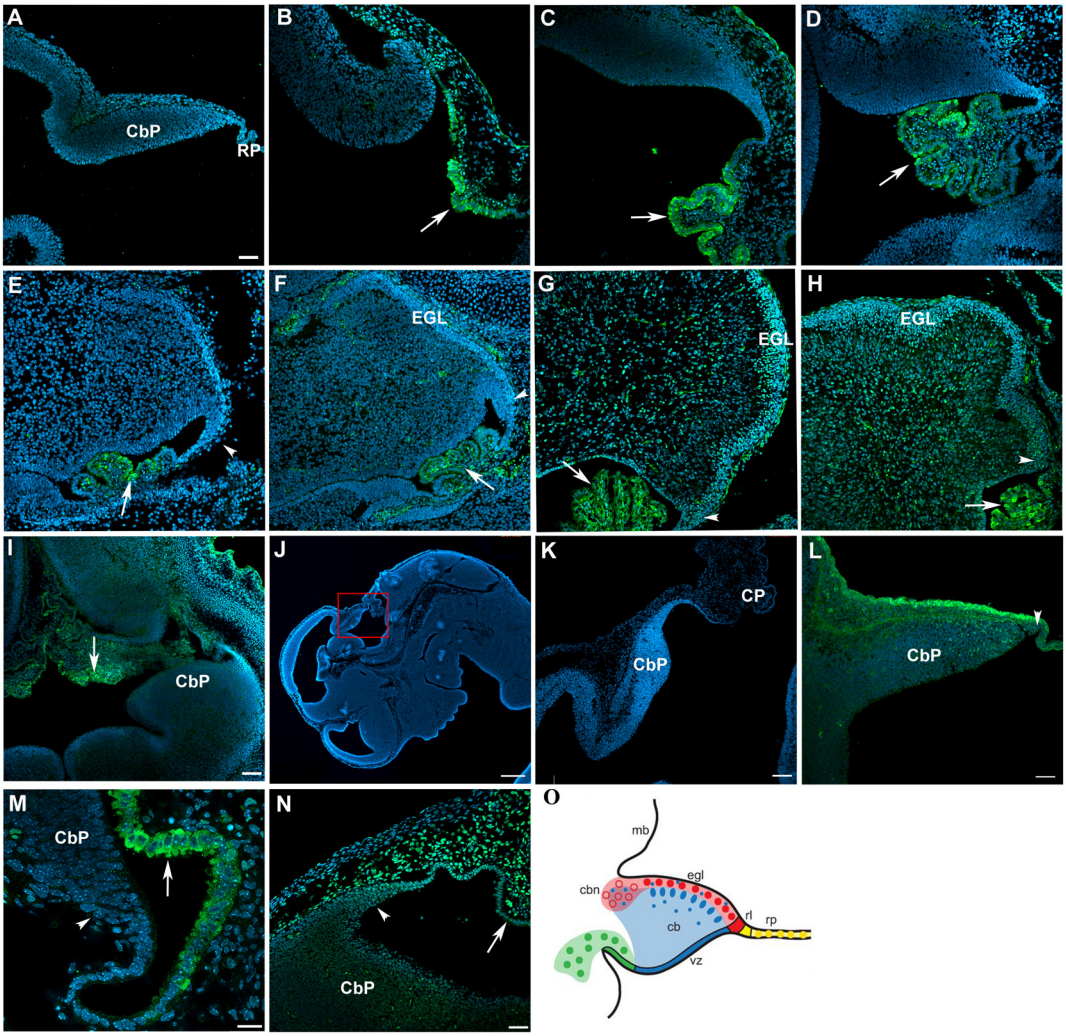

Figure 5. Cux2 (A-I) and Math1 (L) immunohistochemistry in mouse embryos. Cux2 is not expressed at E10 (A; scale bar= 50µm). By E11, Cux2 is in cells clustered in the middle of the RP (B; arrow). Between E12.5-E15.5, Cux2 continues to be expressed in cells in the middle of the developing and invaginating CP, but not in cells of the RL (C-F, respectively; arrows and arrowhead, respectively). However, by E16.5 through E17.5, Cux2 is strongly expressed in the RL, EGL and continues to be present in the CP (G and H, respectively; arrowhead and arrow, respectively). A coronal section at E13.5 reveals that Cux2 is not expressed in the developing cerebellum, but is strongly expressed in the cells in the middle of the developing CP (I; arrow; scale bar= 100µm). A 2x image reveals the developing cerebellum within the entire E10.5 embryo (J; red box; scale bar= 500µm). A 10x image reveals the developing cerebellum and CP within the hindbrain at E11.5 (K; scale bar= 100µm). Math1 expression is in the developing cerebellar primordium and future RL region at E10.5 (L; arrowhead; scale bar= 50µm). A high magnification image image reveals the clear Cux2 expression in the CP and not in the developing RL at E12.5 (M; scale bar= 20µm). A Cux1 antibody was used to show the clear expression of Cux1 in the developing CP and RL, extending beyond the expression of Cux2 protein (N; scale bar=100µm). A model highlighting the major germinal zones and cell types to show orientation of experimental pictures (O). N(A-I)= 2; N(J-L)= 2. CP= Choroid Plexus; RL= Rhombic Lip; EGL= External Granule Layer; CbP= Cerebellar Primordium.

this region. However, the tendency of Cux2-positive cells to be in the middle region of the invaginating CP was consistent at all mediolateral levels. Furthermore, this same sort of clustering was observed in coronal sections with Cux2-expressing cells located in the middle of the developing CP at E13.5 (Fig. 5I).

It was surprising to notice that Cux2 protein expression through E15.5 did not seem to match the expression of its transcripts at early stages during the formation of the cerebellar primordium (e.g. E13.5). However, there was a change by E16.5 such that Cux2 protein and mRNA expression were concordant (Fig. 4 and Fig. 5F-I). At E16.5 Cux2 was strongly expressed throughout the developing CP, but it also expanded into the developing cerebellum (Fig. 5G). Expression was strong in the RL, EGL and in cells migrating inwards from the EGL. In addition, Cux2 protein was expressed in cells deeper in the developing cerebellum. This protein expression pattern was maintained at E17.5 (Fig. 5H). The discordance between *Cux2* mRNA and protein expression has been reported in previous studies that did not detect Cux2 protein expression in all cell lines that expressed *Cux2* mRNA (Gingras et al., 2005). It does however demonstrate the need to focus on mRNA expression data to inform studies of fate map, which ultimately reflect locus activity of the gene of interest.

There are interesting differences between Cux2 expression relative to that of the well known marker for the cerebellar primordium, Math1 (Machold and Fishell, 2005, Wang et al., 2005). When IHC is performed against Math1 in the mouse embryo at E10.5, it clearly labels the developing cerebellar primordium and future RL region (Fig. 5L). This expression highlights a major difference between Math1 and Cux2 expression. Specifically, at E10.5 DCN neurons are being generated from the RL and Math1 is strongly expressed while Cux2 is not yet expressed. Instead the Cux2 expression was activated at much later stages in the cerebellar primordium (E12.5 onwards) coinciding with GC neurogenesis, migration, and maturation.

Altogether, these results highlight that *Cux2* mRNA was strongly expressed in the developing cerebellum throughout neurogenesis in both the mouse and chick. Specifically, it was expressed in regions associated with the generation and maturation of GCs, namely the RL and EGL. The fact that *Cux2* expression was conserved between amniotes and mammals provides more evidence for the high degree of similarity in mechanisms involved in cerebellar development between these animals. Furthermore, these results validate the use of the chick in subsequent studies interested in understanding the role of *Cux2* in cerebellar development.

3.2. Cux2 Fate Mapping

Once it was determined that *Cux2* expression was coincident with the development of GCs, a fate mapping study was performed to determine the fate of this group of cells. There are very few known factors involved in fate restriction in the developing cerebellum, particularly in the RL. Given this, it was very interesting that *Cux2* seemed to be very restricted in expression to the RL and it prompted us to determine whether or not it was restricted in expression to a distinct derivative from this neurogenic region. Furthermore, Cux2 has previously been shown to be fate restricted to hippocampal granule cells, subsets of spinal cord interneurons and upper layer cortical neurons, suggesting its activity in progenitors can act in fate restriction (Cubelos et al., 2008, Iulianella et al., 2008, Iulianella et al., 2009, Franco et al., 2012, Yamada et al., 2015). Therefore, a fate mapping strategy was used to determine if *Cux2* was restricted to a particular cell type in the developing cerebellum. This portion of the study used the previously reported $Cux2^{CreERT2/+}$:$Rosa26^{tdTomato/tdTomato}$ inducible mouse line (Franco et al, 2012). This line contains the human estrogen receptor and thus Tamoxifen, an estrogen mimetic, can bind to this receptor and translocate Cre to the nucleus. Once in the nucleus, Cre causes recombination, resulting in the transcription of tdTomato in cells that transcribe *Cux2* (Fig. 2). This strategy allowed us to label *Cux2*-expressing cells at various points during development to analyze their fate at a later time point.

The first portion of this study involved analyzing $Cux2^{CreERT2/+}$;$Rosa26^{tdTomato/tdTomato}$ animals at E16.5. These animals were dosed with Tamoxifen at E10.5 and E12.5 (Fig. 3). Due to the delay in recombination, these doses labeled cells expressing *Cux2* at E11-12 and E13-14, respectively (Danielian et al., 1998, Zervas et al., 2004). These dosing time points were chosen to include the generation of major cerebellar cell types. For example, between E10-12, DCN neurons are being generated from the RL and PCs are being generated from the VZ (Miale and Sidman, 1961, Goldowitz et al., 1997, Machold and Fishell, 2005, Fink et al., 2006, Morales and Hatten, 2006). Subsequently, after E12.5, GCs and UBCs are being generated from the RL and inhibitory interneurons and glia are being generated from the VZ (Dahmane and Ruiz i Altaba, 1999, Hoshino et al., 2005, Machold and Fishell, 2005, Englund et al., 2006, Chung et al., 2009, Lundell et al., 2009, Consalez and Hawkes, 2012, Leto et al., 2012). Therefore, these two time points would reveal whether *Cux2* was expressed in any cerebellar precursors during early cerebellar neurogenesis.

Animals dosed at E10.5 had labeled cells along the midline that looked like radial glia, and in the developing CP (N=4; Fig. 6A'). In contrast to these cells in the midline and CP, there were very few labeled cells in each developing hemisphere (Fig. 6A). In this group of animals the majority of labeled cells co-expressed Pax6 over Calbindin and NeuN, 36.7% versus 14.6% and 4.1%, respectively (Fig. 7). At this time in development, Pax6 is predominantly expressed in GC progenitors and not in DCN neurons (Engelkamp et al., 1999, Fink et al., 2006, Chung et al., 2010). In addition, Calbindin is expressed in PCs beginning at E14 and NeuN is expressed in a small subset of post-mitotic GCs and DCN neurons at this time (Wassef et al., 1985, Tolosa de Talamoni et al., 1993, Weyer and Schilling, 2003, Fink et al., 2006, Chung et al., 2010). However, there were a large number of cells that did not co-express Pax6, Calbindin or NeuN (44.6%). These tomato-positive cells were likely the cluster of cells along the midline that looked like radial glia that did not co-label with NeuN, Pax6 or Calbindin. The next cohort of animals was dosed with Tamoxifen at E12.5 (N=4). These embryos had many more labeled cells, mostly in the RL and developing EGL (Fig. 6B-F). These cells preferentially co-expressed Pax6 over Calbindin and NeuN, 75.1% versus 9.4% and 17.7%, respectively (Fig. 7). The majority of co-labeling with Pax6 was found in the RL and EGL (Fig. 6B and 6C). Although there was slight co-labeling with Calbindin, the labeled cells seemed to inhabit an exclusive domain from the Calbindin-positive cells (Fig. 6D). Beyond the difference in the percent of Pax6 co-expressing cells, which more than doubled, the major difference between these two dosing time points was the number of labeled cells. Animals dosed at E12.5 had many more labeled cells, suggesting that *Cux2* was expressed more abundantly at E13 over E11. Overall these findings are entirely consistent with the robust expression of *Cux2* in the RL (Fig. 4).

The second phase of this experiment involved analyzing animals at P14, which is the stage when the cerebellum is considered mature. The most abundant GC proliferation has already occurred and most cells have migrated into the GCL, only a few remain in the EGL (Miale and Sidman, 1961, Wallace, 1999, Lewis et al., 2004, Butts et al., 2014). Animals were dosed with Tamoxifen at E10.5, E12.5 and E17.5 (Fig. 3), and the delay in recombination would mean that labeling would occur in cells that expressed *Cux2* at E11-12, E13-14 and E18-19, respectively (Danielian et al., 1998, Zervas et al., 2004). In addition to studying these points for the reasons outlined above, we also focused on a later time point (E17.5) to determine whether *Cux2* was

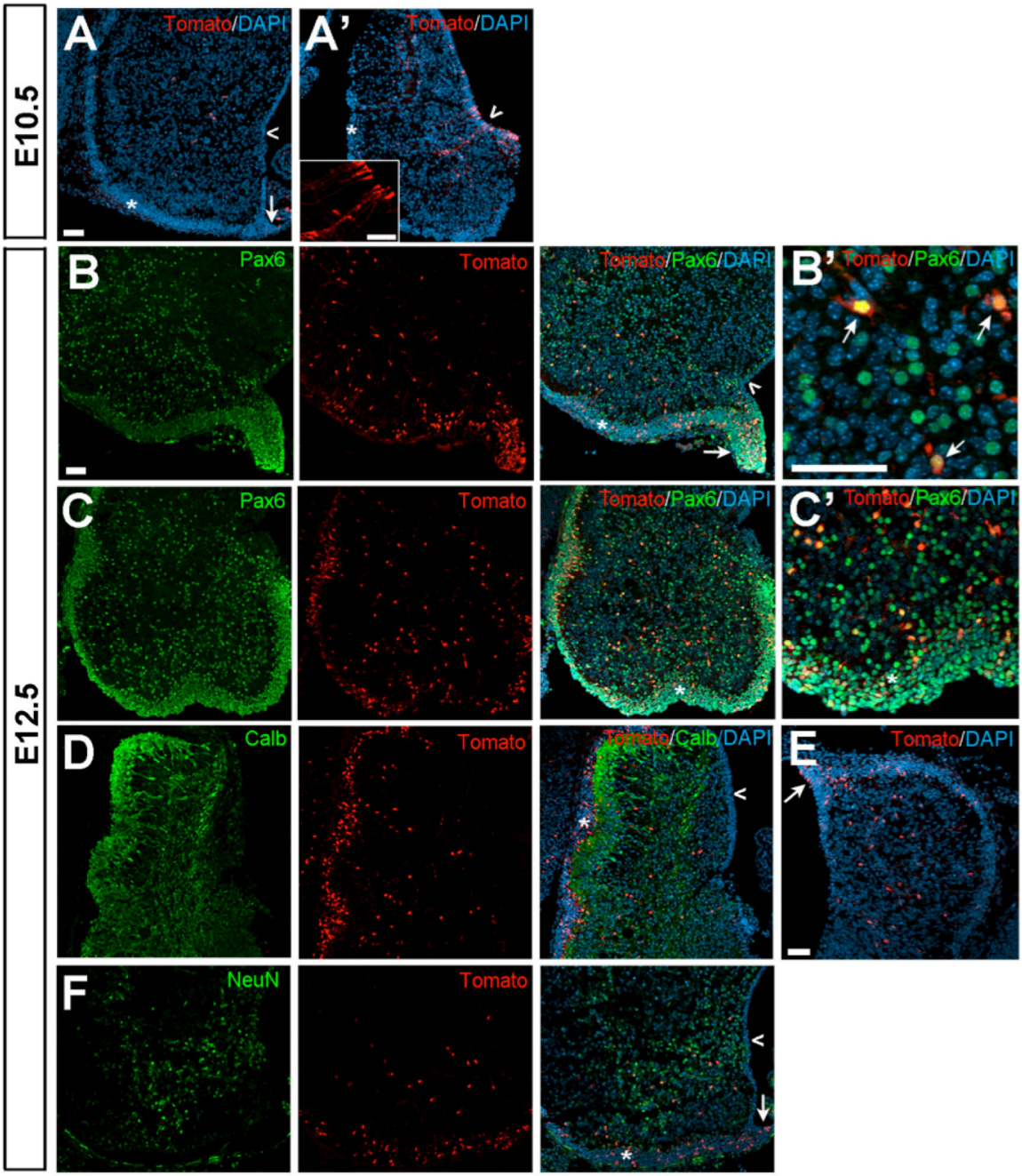

Figure 6. *Cux2*$^{CreERT2/+}$*:Rosa26*$^{tdTomato/tdTomato}$ mice were used and pregnant dams were dosed with 2mg of Tamoxifen to cause genetic recombination and tomato-labeling of *Cux2*-expressing cells. Animals were dosed with Tamoxifen at E10.5 (A and A'; N=4) and E12.5 (B-F; N=4) to reveal the restriction of *Cux2* to GC progenitors when animals were analyzed at E16.5. Dosing at E10.5 reveals very few *Cux2* derivatives in the cerebellar hemispheres, evidenced by the small number of tomato-positive cells, with no tomato-positive cells in the RL (A; arrow). However, these animals do have a cluster of cells in the midline that resemble radial glia (A'). Dosing animals at E12.5 reveals strong co-expression of *Cux2*-derivatives with Pax6 in both the RL (B and B') and EGL (C and C'). In contrast, these animals show very little co-expression with Calbindin (D) or NeuN (F). In addition, E shows the clear localization of *Cux2*-derivatives, labeled with Tomato, in the RL, the site of GC generation (arrow). Arrowheads identify the ventricular zone, while asterisks identify the external granule layer. Scale bars= 50μm.

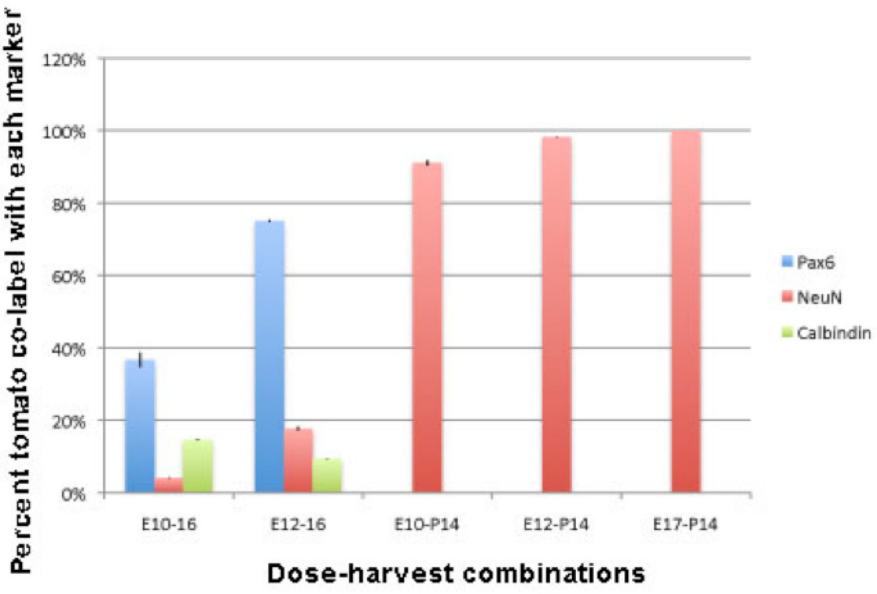

Figure 7. A bar graph showing the proportion of *Cux2*-derived (tomato-positive) cells that co-express Pax6, NeuN and Calbindin at E16.5 and P14. All three of these markers were used to identify cells at E16.5, while only Calbindin and NeuN were used at P14. Animals dosed at E10.5 had the majority of co-labeling with Pax6 at E16.5 and with NeuN at P14. When animals were dosed at E12.5 they had the most labeling with Pax6 at E16.5 and with NeuN at P14. Finally, animals dosed at E17.5 had exclusive co-labeling with NeuN at P14. Variance is shown for each proportion. N=4 for each dose-harvest combination.

expressed late in cerebellar neurogenesis encompassing the proliferation of GCs and migrating UBCs (Smeyne et al., 1995, Dahmane and Ruiz i Altaba, 1999, Wallace, 1999, Lewis et al., 2004, Englund et al., 2006, Chung et al., 2009). Analyzing animals at P14 allowed us to verify and compare results with animals analyzed at E16.5, to determine if results were consistent throughout development.

When animals were dosed with Tamoxifen at E10.5, there was minimal labeling throughout the cerebellum and 91.1% of tomato-positive cells co-labeled with NeuN, versus the 0.1% that co-labeled with Calbindin (N=4; Fig. 7). At P14, Calbindin strongly labels PCs (Wassef et al., 1985, Tolosa de Talamoni et al., 1993). In contrast, NeuN is expressed in DCN neurons and in the cortex it is restricted to GCs. Therefore, if tomato-positive cells are found in the cortex co-labeled with NeuN then they are GCs, and not cells of another interneuron population (Weyer and Schilling, 2003, Fink et al., 2006). The majority of regions had a few labeled cells, with a couple of cells that looked like Bergmann glia and a couple that looked like developing GCs (Fig. 8A). The next cohort of animals was dosed at E12.5 (N=4). Again, there was a dramatic difference in the number of labeled cells between these two early dosing time points. Animals dosed at E12.5 often had labeled cells that comprised multiple entire folia in a section (Fig. 8B). Dosing at this time point labeled cells that most prominently co-expressed NeuN with 98.2% of cells co-expressing this marker, versus 0.1% that co-labeled with Calbindin (Fig. 9B and 7). Some axial levels had few or no labeled cells, but this was less common than at E10.5. Labeled cells were most commonly clustered, filling half a folium, an entire folium or multiple adjacent folia with a continuous pattern (Fig. 10A and 10A'). The final group of animals was dosed with Tamoxifen at E17.5 (N=4). This cohort had a similar amount of labeling to animals dosed at E12.5 and labeled cells were found in similar clustered patterns (Fig. 10B and B'). Again, *Cux2*-tomato labeled cells exclusively co-expressed NeuN (100%) and not Calbindin (0%; Fig. 7 and 8C). Overall, very few cells co-expressed Calbindin, with only 1-2 cells observed in animals dosed at E10.5 and E12.5 and no visible cells co-labeled in animals dosed at E17.5 (Fig. 9A-C), demonstrating Cux2 almost exclusively fate mapped to cerebellar GCs.

Interestingly, although P14 animals dosed at E12.5 and E17.5 had similar levels of labeling found in clusters, expression patterns seemed to be the opposite (N=4; Fig. 10A and B). For example, animals dosed at E12.5 had the majority of labeling in posterior folia, while

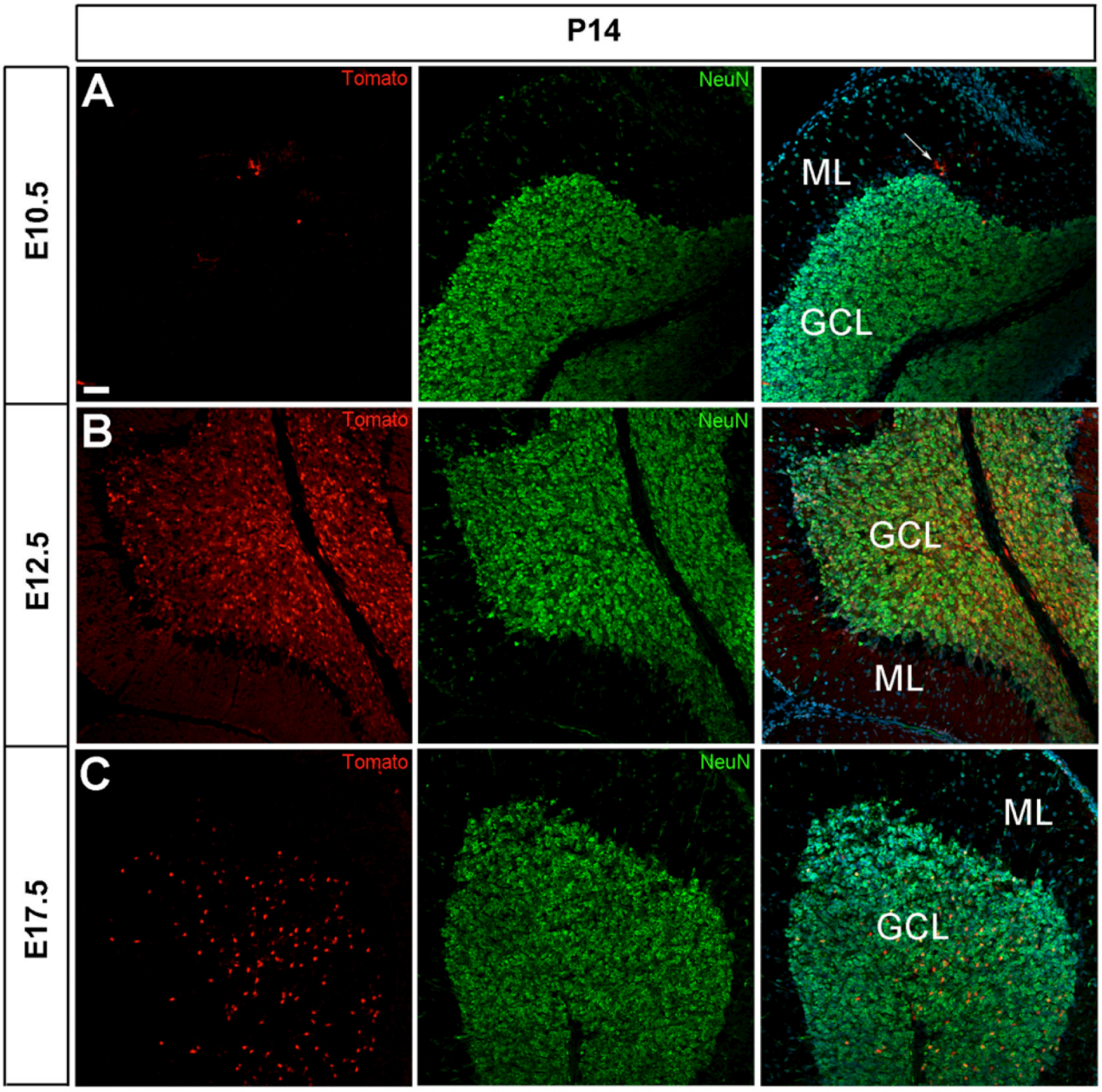

Figure 8. *Cux2*$^{CreERT2/+}$*:Rosa26*$^{tdTomato/tdTomato}$ animals were dosed with Tamoxifen at E10.5, E12.5 and E17.5 and analyzed at P14. Animals dosed at E10.5 have minimal labeling, with a couple of cells that look like glia and some cells in the GCL that co-label with NeuN (A; arrow). In contrast, animals dosed at E12.5 had abundant labeling throughout the GCL with exclusive co-labeling with NeuN (B). In addition, animals dosed at E17.5 had a lot of labeling in the GCL and exclusive co-expression with NeuN (C). ML= Molecular layer; GCL= Granule Cell Layer; asterisks= external granule layer. N=4 for each dose time point. Scale bar= 50µm.

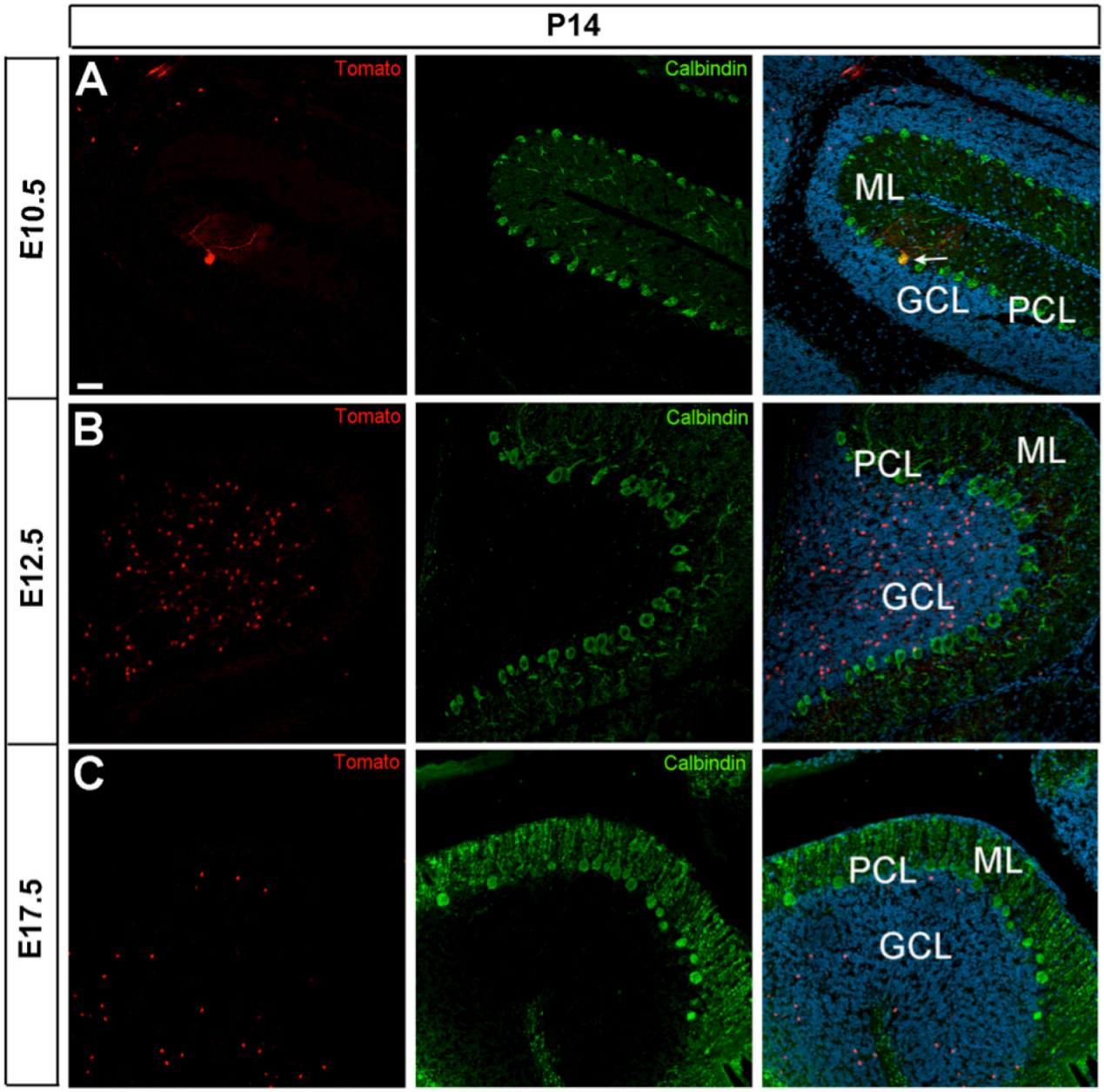

Figure 9. *Cux2*$^{CreERT2/+}$*:Rosa26*$^{tdTomato/tdTomato}$ animals were dosed with Tamoxifen at E10.5, E12.5 and E17.5 and analyzed at P14. Animals dosed at E10.5 had some labeled cells that were mostly found in the GCL; however there were a couple of PCs that were tomato-labeled that co-expressed Calbindin (A; arrow). When animals were dosed at E12.5 there was abundant tomato-labeling in the GCL and almost no co-labeling with Calbindin (B). Finally, when animals were dosed at E17.5 there were a lot of labeled cells in the GCL with no co-labeling with Calbindin (C). ML= Molecular Layer; PCL= Purkinje Cell Layer; GCL= Granule Cell Layer. Scale bar= 50µm.

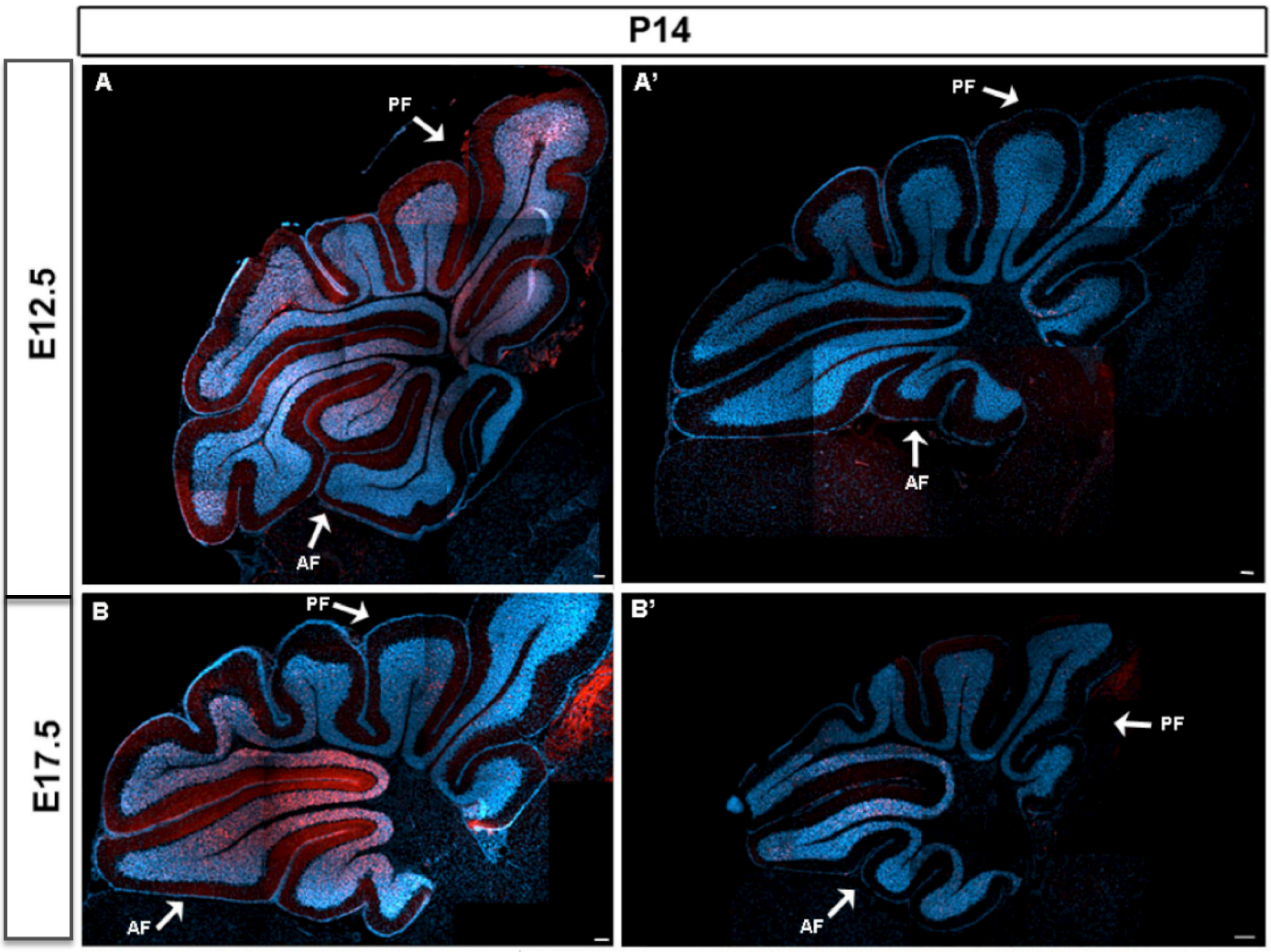

Figure 10. When *Cux2*$^{CreERT2/+}$:*Rosa26*$^{tdTomato/tdTomato}$ mice were dosed with Tamoxifen at E12.5 and E17.5, there was strong variability in both the mediolateral and anteropeosterior directions. When animals were dosed at E10.5 there were some sections with a large number of labeled cells (A), while other axial levels has almost no labeling (A'). In addition, these animals seemed to have stronger labeling in the PF compared to the AF, even when there was minimal labeling (A and A'; arrows). In contrast, when animals were dosed with Tamoxifen at E17.5 there was preferential labeling in the AF compared to the PF, at all axial levels (B and B'; arrows). In addition, these animals also had abundant variability in the mediolateral direction, with some levels having a lot of labeled cells (B) and others having very few labeled cells (B'). PF= Posterior Folia; AF= Anterior Folia. N=4 for each dose time point. Scale bars= 100µm.

animals dosed at E17.5 had the majority of labeling in anterior folia (Fig. 10A and B). This was a consistent trend for animals within each cohort; however, there was still a substantial amount of variability in the mediolateral direction within each cohort. For example, at some levels half a folium at the rostral or caudal edge of the cerebellum was tomato-positive, while at other levels the entire cerebellum was labeled with an obvious gradient from rostral to caudal or vice versa (Fig. 10A-B'). This pattern may be simply reflecting the gradient of GC neurogenesis and then maturation (Machold and Fishell, 2005; Wingate and Hatten, 1999). Furthermore, although expression at different axial levels varies in the GCL expression, there is consistently no labeling of DCN (Fig. 11C).

When animals were analyzed at P14 after being dosed at E10.5 and E12.5 there was also significant labeling in the choroid plexus (CP; Fig. 11A and B). This labeling was seen in each animal.

3.3. *Cux2* Electroporations *in ovo*

In an attempt to begin understanding the role of *Cux2* in cerebellar development, an overexpression study was performed. Many of the seminal studies investigating the origins of the cerebellum and its germinal zones were completed in the chick, validating it as a model system for cerebellar development (Hallonet et al., 1990; Hallonet and Le Douarin, 1993; Rhinn and Brand, 2001; Martinez and Alvarado-Mallart, 1989; Alvarez Otero et al., 1993; Hidalgo-Sanchez et al., 2005). Other studies have also revealed that mechanisms controlling cerebellar development in the mouse are similar to those governing development in the chick (Butts et al., 2014, Green and Wingate, 2014). Specifically, in the chick GCs arise from the RL around E6, consistent with studies in the mouse in which they arise around E12.5 from the same region (Ryder and Cepko, 1994, Wingate and Hatten, 1999, Machold and Fishell, 2005). In addition, previous studies have used this technique to determine the effects of *Cux2* overexpression. For example, *Cux2* overexpression in the chick spinal cord or olfactory epithelium revealed an important role of *Cux2* in the proliferation and maturation of distinct subpopulations of neurons. These studies have validated the use of the chick model for *in ovo* electroporations of *Cux2* to determine gene function (Iulianella, 2008; Wittmann, 2014). *Cux2pCIG* and *pCIG* control vectors were prepared at 3.7 μg/μl in the pCAAGS expression vector, which harbours the

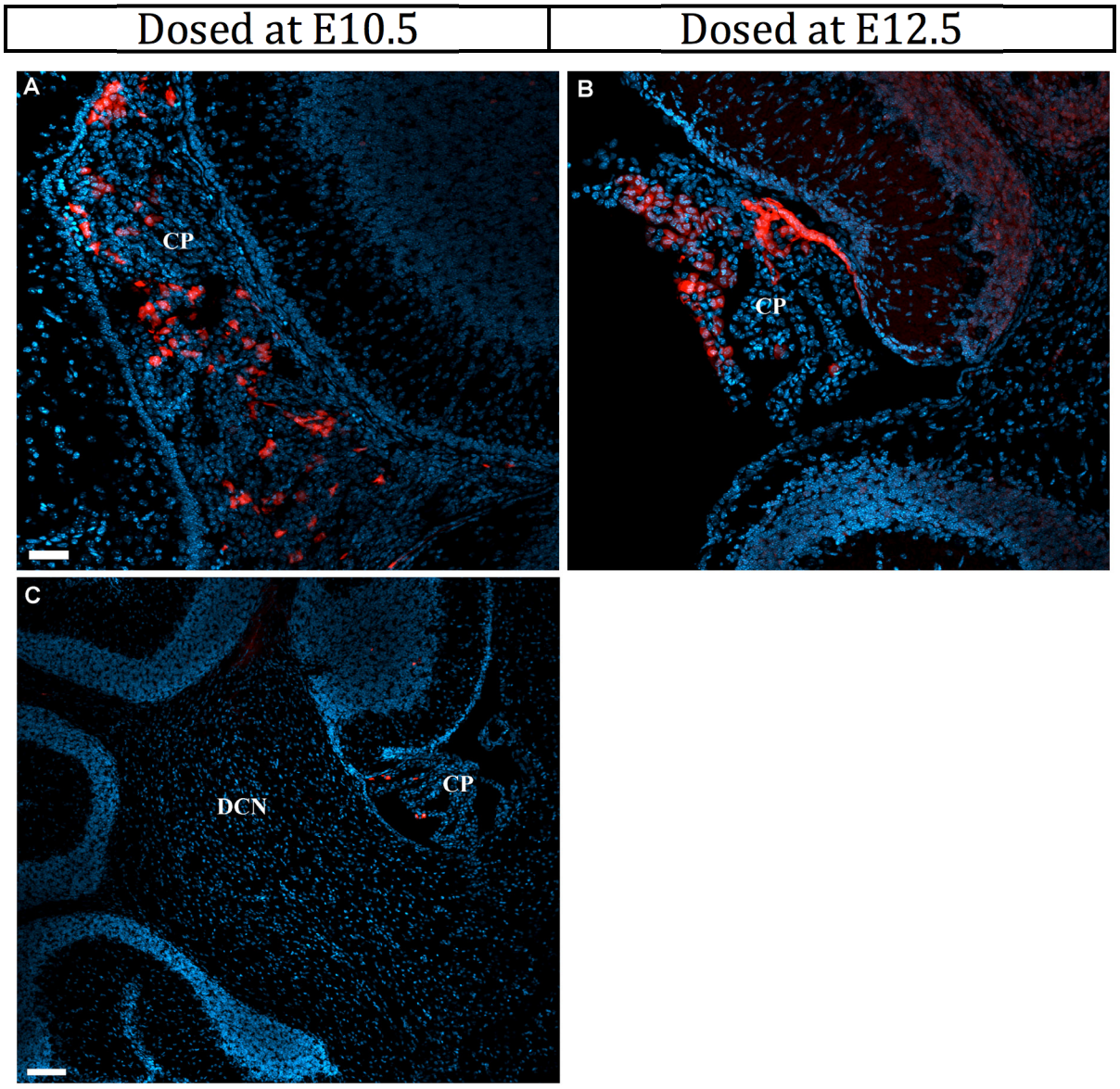

Figure 11. When *Cux2*$^{CreERT2/+}$:*Rosa26*$^{tdTomato/tdTomato}$ mice were dosed with Tamoxifen at E10.5 and E12.5 and analyzed at P14, the choroid plexus was labeled with Tomato (scale bar= 50µm). A low magnification view of the white matter at P14 in an animal dosed at E10.5 reveals the absence of Tomato-labeled cells in the DCN (C; scale bar= 500µm). CP= Choroid plexus.

powerful ubiquitous promoters CMV and beta-actin, as previously described (Wittmann et al., 2014). *Cux2pCIG* and control plasmids were electroporated unilaterally into the developing hindbrain of E2 chicks (HH stage 11-13; N=6 experimental and N=4 control), and embryos we allowed to develop until E9 (HH stage 35). By E9, GCs have been generated for a few days and are actively migrating up and over the developing primordium (Hallonet et al., 1990, Ryder and Cepko, 1994, Lin and Cepko, 1998, Wingate and Hatten, 1999, Lin et al., 2001). Electroporated animals were sectioned parasagittally and analyzed with NeuN, Pax6 and Calbindin to identify post-mitotic DCN neurons and GCs, GC precursors and PCs, respectively (Wassef et al., 1985, Tolosa de Talamoni et al., 1993, Weyer and Schilling, 2003, Fink et al., 2006). An immunohistochemistry experiment using the Cux2 antibody verified that there was no overexpression in control animals (12J-J'') and abundant Cux2 expression in experimental animals (12K-K''). The number of cells co-localized with each marker was counted, along with the total number of eGFP-positive cells in each section. From these counts we identified a % co-localization of eGFP-positive cells with NeuN, Pax6 and Calbindin for each animal and averaged for each experimental group. Examples of the co-expression patterns are shown for both the experimental and control groups (Fig. 12A-F'). From this analysis it was found that at E2 *Cux2* alone was unable to significantly alter the fate of the RL progenitors. Specifically, both animals had the same proportion of *eGFP*-positive cells that co-labeled with Pax6, NeuN and Calbindin (Fig. 12I). The lowest proportion of electroporated cells co-expressed Pax6, while the highest proportion of cells co-expressed NeuN and co-expression of Calbindin was in between the former 2 markers (Fig. 12I). These results suggest that by itself, *Cux2* is unable to promote the generation of GCs from early hindbrain progenitor pools.

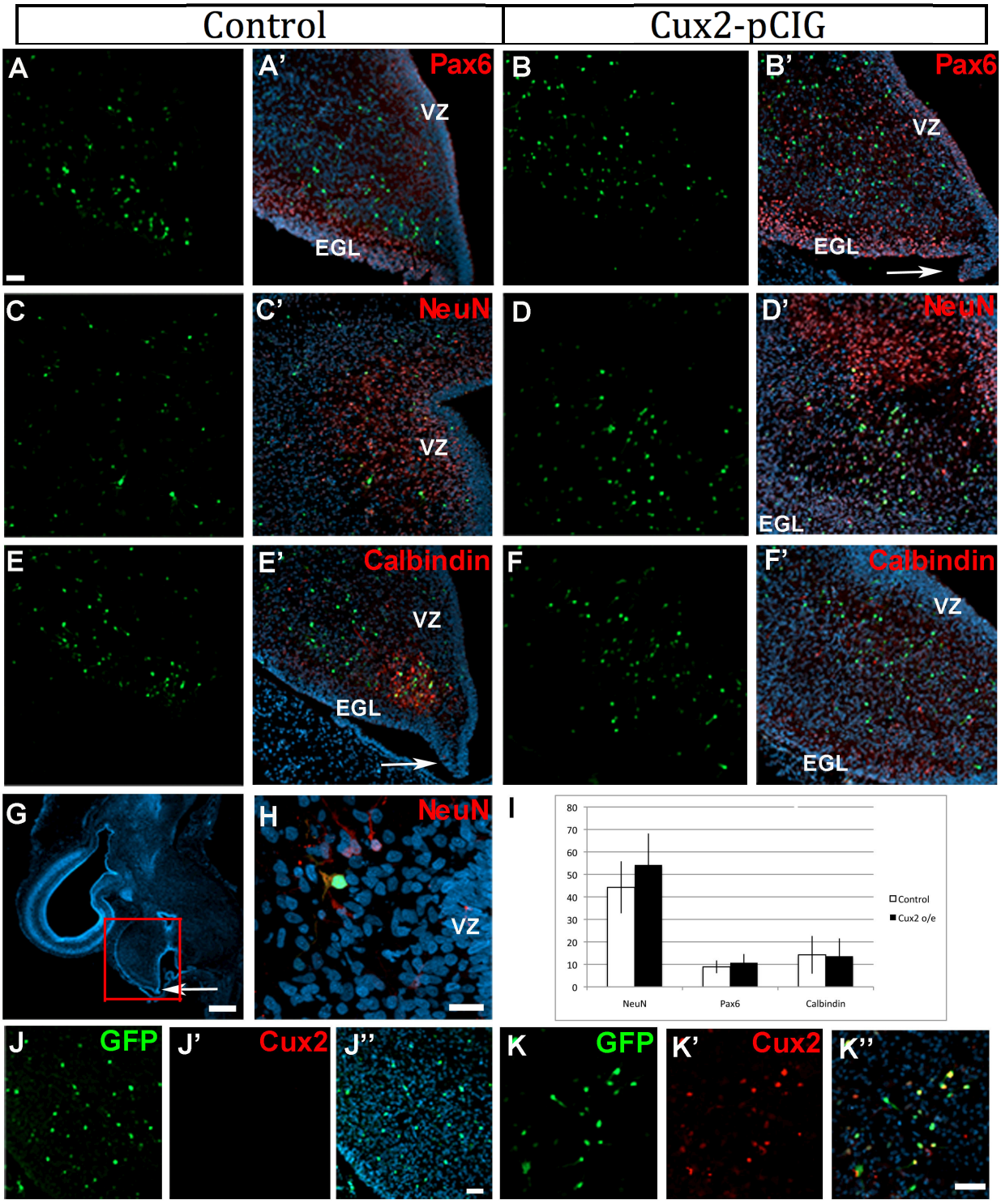

Figure 12. Chick embryos were electroporated at E2 with a *Cux2*-pCIG (N=6) or control (N=4) plasmid. Animals were analyzed at E9. Both groups of electroporated animals co-labeled minimally with Pax6 (A-B'; scale bar=50μm). About half of the electroporated cells co-labeled with NeuN (C-D' and H; scale bar=20μm). Finally, both groups had few cells that co-expressed the plasmid and Calbindin (E-F'). A low magnification image shows the region in which the counts were taken from in the developing cerebellum (G; red box; Scale bar= 100μm). The rhombic lip is also shown (B' and E'; arrows). Counts were average within each group for NeuN, Pax6 and Calbindin and the variances are shown (I). mCux2 antibody staining shows no co-localization with the control plasmid (J-J'') and abundant co-localization with the *Cux2*-plasmid (K-K''; scale bar= 100 μm). EGL= External Granule Layer; VZ= Ventricular Zone.

Chapter 4: Discussion

A major question in developmental neurobiology concerns the diversity of cell types in the brain. Even focusing on a presumably developmentally simple region, such as the RL primordium, the degree of diversity of cell types is largely unknown. For instance, it is not known if cerebellar GCs constitute a single type of cell or whether there are subclasses of GCs, each with their unique cell lineage, connectivity and physiological properties. A first step to address this issue requires the development of novel lineage markers for the RL and its derivatives. Based on our previous finding that the transcription factor Cux2 is expressed in a subpopulation of hippocampal progenitors that give rise to granule cells (Yamada et al., 2015), we investigated whether it also acts within the RL progenitors to specify a GC identity.

Cux2 is discretely expressed in the nervous system during development. For example, it is expressed in a specific subset of progenitors that will give rise to upper cortical layer neurons, in non-proliferative granule cell precursors of the hippocampus and in subsets of spinal cord interneurons, among other cell types in other regions (Nieto et al., 2004, Iulianella et al., 2008, Yamada et al., 2015). These previous studies highlight that Cux2 is likely involved in the functions of distinct populations in the brain regions in which it is expressed. This suggests that if Cux2 is expressed in the cerebellum then it is likely restricted to a distinct region and is active in a subpopulation of cells derived from that region. In addition, the mechanisms involved in progenitor cell fate restriction during cerebellar development are still largely a mystery. Therefore, experiments were designed in order to investigate the expression pattern of Cux2 during development to determine whether it is restricted to a single population or subpopulation of cells. In addition, the final experiments were conducted to provide an insight into the possible function of *Cux2* in cell fate restriction. It was found that during development *Cux2* was expressed in the germinal zone of excitatory cerebellar neurons during the generation of GCs. In contrast, Cux2 protein was not found in this region, but was restricted to the developing CP early in development. Furthermore, fate mapping analyses revealed that *Cux2* was restricted to expression in GC progenitors. Finally, there was not a significant effect of *Cux2* overexpression on fate determination. Overall, *Cux2* was found to be restricted to expression in GC germinal zones and in GC progenitors throughout development, but that in itself was insufficient to markedly influence GC differentiation in overexpression assays of naïve hindbrain tissue.

4.1. *Cux2* Expression was Restricted to Sites of Granule Cell Development

To better understand the role of *Cux2* during cerebellar development, we first characterized its expression pattern during the formation of the RL and cerebellar primordium. We wanted to know if the expression during development was restricted to a discrete germinal region and what the developmental period was for expression. *Cux2* was expressed early during development in the chick. At E2 (HH 9-11), *Cux2* expression was detected in the developing chicken midbrain and at the junction of the midbrain-hindbrain and midbrain-forebrain. Interestingly, the junction of the midbrain-hindbrain has established a rostral expression of *Otx2* and a caudal expression of *Gbx2* at this point in development (Rhinn and Brand, 2001, Hidalgo-Sanchez et al., 2005). The *Fgf8*- and *Otx2*-positive rostral domain and *Irx2*- and *Gbx2*-positive caudal domain molecularly delineate the MHO (Rhinn and Brand, 2001, Matsumoto et al., 2004, Hidalgo-Sanchez et al., 2005). However, *Cux2* was expressed throughout the entire midbrain-hindbrain junction in regions both rostral and caudal to the MHO. Since *Cux2* was expressed in a broader domain than the presumptive hindbrain-forming region, it unlikely plays an essential role in the patterning of the MHO. In addition, *Cux2* was most strongly expressed in the dorsal portion of the interior neural tube. The dorsal portion of the neural tube includes the cerebellar primordium. At E2, the RL can include cells from the entire dorsal two-thirds of the neural tube, meaning that cells from this region will give rise to each RL derivative, including GCs (Wingate and Hatten, 1999). Therefore, early in chick development *Cux2* was highly expressed in the region that gives rise to the developing hindbrain, and specifically, the RL and its derivatives.

Cux2 expression was also observed later in the GC precursor region of the developing cerebellum in both the chick and the mouse. Specifically, in the mouse at E13.5, *Cux2* was strongly expressed in the RL, the site of GC generation (Machold and Fishell, 2005, Leto et al., 2012). At this time, *Wls, Math1,* and *Pax6* are also expressed in the RL and are important for GC generation (Machold and Fishell, 2005, Fink et al., 2006, Yeung et al., 2014). Furthermore, in the chick at E9 and E10 (HH stages 35 and 36, respectively) *Cux2* was strongly expressed in the RL and EGL. In addition, at E10 (HH stage 36) there was a more ventral lighter band of cells that was anatomically similar to the GCL. For example, this deeper layer had small cell bodies and was a thin band separated from the EGL, characteristics of the GCL (Martinez et al., 2013). All of these results highlight the distinct *Cux2* expression in the RL and EGL, sites of GC generation and proliferation.

The restricted expression of *Cux2* to the RL and EGL of the developing cerebellum suggests that it plays a role in the cells that are generated from this region. At E13.5 in the mouse, this region is actively generating GC precursors that are migrating up and over the developing primordium (Machold and Fishell, 2005, Leto et al., 2012). While in the mouse, the tangentially migrating GC progenitors arise from the RL around E12.5, it is around E6 (HH stages 28-30) in the chicken that GC precursors arise from the equivalent region, revealing that in the chick *Cux2* expression was maintained well beyond the onset of GC generation (Hallonet et al., 1990). Thus, *Cux2* was expressed during the initial generation of GCs from the RL and expression was maintained even after these cells had migrated into the EGL. This highlights the importance of prolonged expression of *Cux2* for GC generation and development.

Due to the complexity of GC development, many mechanisms and factors are important in regulating developmental processes of these cells and are thus restricted to expression in this region. For example, *Math1* and *Pax6* are both restricted in the developing cerebellum to expression in the RL and its derivatives and both play crucial roles in neurogenesis in this region. *Pax6* is essential for the neurogenesis of GCs and for formation of the EGL and mature folia (Engelkamp et al., 1999, Swanson et al., 2005, Swanson and Goldowitz, 2011). In addition, Pax6 is thought to regulate *Math1* expression, and also function downstream of *Math1*, highlighting an important co-dependence and feedback regulation of RL factors in the proper development of this region (Wang et al., 2005, Englund et al., 2006, Fink et al., 2006, Yeung et al., 2014). Although *Math1* has been shown to be essential in GC development, it also has a key role prior to this population being generated. It is essential in the initial generation of the RL and in the generation of other RL derivatives (Ben-Arie et al., 1997, Machold and Fishell, 2005, Wang et al., 2005). Therefore, although both of these factors are essential in generating GCs, *Pax6* is important for their generation and early maturation, while *Math1* being more important for the early patterning of this region. These factors highlight the importance of regional specificity in mRNA and protein expression, as this patterns the developing cerebellum and its germinal zones. This hints at the potential importance of restricted *Cux2* expression for cerebellar specification and development.

4.2. *Cux2* Expression Occurs in Stripes During Late Cerebellar Development

The expression of *Cux2* in the EGL from E9-11 (HH stages 35-37) in the chick was found in a striped pattern. *Cux2* transcripts were detected in two single bands at E9 and 4 bands at E10. By E11, these 4 bands were even more distinct and expression was obvious in the EGL. This expression pattern was reminiscent of the expression of many other genes at this time point in the chick. For example, at E8.5 *EphA5*, *Delta1* and *Gli2/4* all have similar rostrocaudal striped patterns on the dorsal surface of the developing anlage. By the following day, *En1, Shh*, and *BMP7* all share this same striped expression. Interestingly, at E8.5, the expression of *EphA5* shares the same single rostrocaudal stripe, complimented with a single mediolateral stripe, similar to the pattern of *Cux2* expression at E9. Furthermore, the expression of *En1*, *Shh* and *BMP7* all share the heterogeneous expression throughout their stripes, similar to the expression of *Cux2*. For example, expression seems to be highest at the tops of developing folia and lighter or absent in the creases between folia (Lin and Cepko, 1999, Wang and Zoghbi, 2001). This expression pattern of *En2* and *Bmp7* is also observed in mice at E17.5 (Millen et al., 1995). *En2* is important in early hindbrain patterning, but also in later stage cerebellar patterning (Dahmane and Ruiz i Altaba, 1999, Wang and Zoghbi, 2001, Sillitoe et al., 2010). Specifically, the distinct striped pattern of this gene is found in PCs, DCN and GCs and is essential for the establishment of a compartmentalized cerebellum that has functional units (Baader et al., 1999, Wang and Zoghbi, 2001, Sillitoe et al., 2010). For example, if *En2* is ectopically expressed it aberrantly affects the expression patterns of subsequent factors important in compartmentalization and results in ectopic afferent fiber projection patterns (Baader et al., 1999, Sillitoe et al., 2010). These results suggest that molecules that exhibit parasagittal patterns of expression can potentially be involved in establishing the compartmentalization that is characteristic of the adult cerebellum. Many of the studies conducted on compartmentalization of the cerebellum have been interested in PC development, as they have been highly implicated in patterning these functional units (Wang and Zoghbi, 2001, Fujita et al., 2012). However, more recent studies suggest that GCs can, in fact, also be subdivided into distinct parasagittal units (Consalez and Hawkes, 2012). Given the segmented expression of *Cux2* along the anterior-posterior extent of the cerebellum, it is tempeting to speculate that *Cux2* plays a role in establishing the boundaries between functionally unique GC populations and thus creates adjacent, yet molecularly distinct, domains. For example, if *Cux2* proves to delineate subpopulations of GCs, then gain of function and loss

of function experiments would result in disproportionate sizes of these subpopulations compared to other non-*Cux2*-expressing cells. However, there are still not many reliable markers of GC subpopulations, making it technically difficult to compare subpopulation sizes. In contrast, if *Cux2* is important in the organization of all GCs, then *Cux2* overexpression would result in an increase in the entire GC population compared with controls.

4.3. Restricted Expression of Cux2 Protein in the Hindbrain During Development

The next step to better understand the role of Cux2 during cerebellar development involved characterizing the localization of its protein expression during the time period in which the cerebellum is giving rise to all major cell types. *Cux2* mRNA was expressed in the regions that give rise to GCs, highlighting an interesting restricted expression pattern and possible role in the generation of this cell population. This study was important to determine whether the protein and mRNA had similar expression patterns in the regions associated with GC development. The fate mapping analyses used a genetic mouse that labeled cells expressing *Cux2*, thus while protein distribution may inform us as to which cells are undergoing recombination, ultimately the best read-out of locus activity is mRNA localization.

The immunohistochemistry for Cux2 was performed at each embryonic day from E10-17 during mouse development. This is the period in which the RL is giving rise to all of its cerebellar derivatives, and PCs, interneurons and glia are generated from the VZ (Machold and Fishell, 2005, Morales and Hatten, 2006). Surprisingly, the expression pattern of Cux2 protein did not exactly match the mRNA expression throughout development. Cux2 protein only began being expressed in the developing dorsal hindbrain at E11.5 and at this time it was restricted to expression in the developing RP. This expression pattern was maintained through E15.5. Finally, at E16.5 the expression of Cux2 protein expanded into the developing cerebellum, with the most abundant expression in the EGL and RL. The expression pattern at E16.5 and E17.5 was therefore consistent with the mRNA expression in the RL at E13.5, with Cux2 highly expressed in sites of GC generation and development. Furthermore, the expression of Cux2 in the CP was consistent with fate mapping studies that revealed tomato-labeled cells in the CP of animals dosed with Tamoxifen at both E10.5 and E12.5 when they were analyzed at P14 (Fig. 11A and B). Thus, altogether, the majority of late Cux2 protein levels nicely correlated with Tomato fate mapped GCs of the developing cerebellum. The only difference occurred early in cerebellar

development, when *Cux2* was expressed in the RL region, which did not express the protein. The reason for this difference is not completely understood, but may involve posttranscriptional mechanisms of *Cux2* control. For instance, Gingras et al (2005) previously noted that while almost all commonly used cell lines expressed *Cux2*, only one line expressed the protein. This suggests that the levels of Cux2 may need to be tightly controlled to ensure proper GC neurogenesis, as was discovered for various neuronal populations of the spinal cord (Iulianella et al., 2008, Iulianella et al., 2009), cortex (Cubelos et al, 2008), and olfactory epithelium (Wittmann et al, 2014). In contrast to the limited expression of Cux2 in the RP and CP until E16.5, Cux1 was strongly expressed throughout the developing cerebellar primordium at E12.5. These results reveal a possibility that Cux1 and Cux2 have over-lapping functions in the developing hindbrain early in development. Overall, these experiments revealed a strong and restricted expression of Cux2 protein in the developing RP and CP.

The RP and CP are hindbrain derivatives that have been under a lot of investigation lately due to their important role in hindbrain patterning and neurogenesis (Alder et al., 1999, Emerich et al., 2005, Chizhikov et al., 2006, Hunter and Dymecki, 2007). From E10.5-12.5, when Cux2 was first detected in the RP and CP, other RP markers such as *Lmx1a* and *Gdf7* are excluded from the *Math1*-positive RL domain, thereby establishing a molecular code differentiating between the RP and CP co-lineage from the RL progenitors. This discrete molecular expression pattern suggests that these two tissues are distinct cellular domains, yet they remain anatomically connected. The RL disappears in late embryonic development, leaving the CP directly connected to the cerebellum in post-natal life, although the two regions serve very discrete functions (Chizhikov et al., 2006, Yeung et al., 2014). Recent studies suggest that the RP and CP both derive from a common progenitor pool of dorsal neuroectodermal precursors within the RL. The neural tube closes transiently at the hindbrain level at E9 in the mouse. However, rather than completely sealing closed, the dorsal hindbrain re-opens as a result of the forward flexing neural tube. As the neural tube re-opens, RP epithelial cells fill in to cover the developing 4th ventricle. The first cohort of RP cells is born between E8-9.5 from a Wnt1-expressing and *Gdf7*-non-expressing region of the RL and they remain proliferative until E10.5. The rest of the RP fills in at E9.5 from RL progenitors that express both Wnt1 and *Gdf7* and are no longer mitotically active once they leave the RL. This later-born group of cells are able to give rise to CP through linear progression and cell shape change beginning around E10 (Hunter and Dymecki, 2007). In

addition, CP cells can derive directly from the RL between E12.5-14, after the disappearance of the RP (Hunter and Dymecki, 2007). These results highlight that the Cux2-expressing cells in the CP were likely RL-derived, in line with the mRNA expression of *Cux2* in the RL at E13.5. Therefore, although it seemed surprising to see Cux2 protein expressed in the developing RP and CP, and not in the RL, these tissues have both been shown to derive from the RL, where *Cux2* was expressed, supporting evidence of a common lineage.

The discrete expression of Cux2 in the RP and CP raises a couple of important possibilities to consider, including that (1) the Cux2 antibody lacks the sensitivity to detect low protein levels in the RL or (2) there is not translation of *Cux2* in every cell that expresses the mRNA, which results in mRNA and protein not always being expressed in the same cells. First, it is possible that the Cux2 antibody is not sensitive enough and that Cux2 protein is, in fact, present in the RL and developing EGL earlier than E16.5. In support of the latter possibility, it has been reported that many glial and neuronal cell lines do not express Cux2 protein, even though they express *Cux2*. Furthermore, a recent study on Cux2 fate mapping in the cortex has also concluding that one cannot guarantee that protein is actually present simply based on mRNA and Cre recombination (Guo et al., 2013). Therefore, it is possible that mRNA and protein are not expressed in the same populations, suggesting posttranscriptional regulation of *Cux2* (Gingras et al., 2005). In summary, my results suggest that while Cux2 protein was restricted to the developing RF and CP, its mRNA was still expressed in the RL and developing anlage earlier than E16.5. Therefore, genetic fate mapping strategies would label derivatives from both of these cell populations since it is dependent on the transcription of *Cux2*, not the presence of Cux2 protein.

4.4. *Cux2* Fate Mapping

The results suggesting the restricted expression of *Cux2* in the regions associated with GC development stood out and prompted us to perform more experiments. Recent studies have provided insight into the mechanisms and factors controlling how the RL progenitors are able to give rise to multiple distinct cell types and into identifying whether or not all of these cells come from common progenitors. The most obvious and consistent expression was in the RL, which generates multiple cerebellar cell types, including DCN neurons, GCs and UBCs (Machold and Fishell, 2005, Leto et al., 2012). Therefore, since *Cux2* was expressed over multiple time points

in both the mouse and chick in a region that sequentially gives rise to multiple cell types it was unclear whether *Cux2* expression was restricted to a single progenitor cell type or was expressed in a multipotent cerebellar progenitor capable of giving rise to all RL derivatives.

In order to understand whether or not *Cux2* was fate restricted, the second part of this study involved fate mapping *Cux2*-expressing cells using a previously described inducible Cre recombinase line driven off the Cux2 genomic region: this line is referred to as $Cux2^{CreERT2/+}$:$Rosa26^{tdTomato/tdTomato}$ (Franco et al, 2012). These mice contain an estrogen receptor ligand-binding domain fused to Cre recombinase to allow genetic recombination of a reporter gene by the administration of Tamoxifen, an estrogen mimetic. The dosing time points fell between E10.5-E17.5 to include the generation of all RL derivatives (Fig. 3). For example, the first-born neurons from the *Math1*-expressing RL are the DCN neurons between E10.5-12.5. Subsequently, between E12.5-17.5 GCs are born from the RL (Machold and Fishell, 2005). The final cell population generated are the UBCs between E15-P2 (Abbott and Jacobowitz, 1995, Sekerkova et al., 2004, Englund et al., 2006, Chung et al., 2009). Therefore, dosing time points were E10.5, which included the generation of DCN neurons from the RL and the generation of PCs from the VZ. The next dosing time point was E12.5, which is the stage at which GCs are generated rapidly from the RL. The final dosing time point was E17.5, at which point GCs are proliferating rapidly in the EGL and UBCs are generated from the RL. The first part of this study involved dosing pregnant animals at E10.5 and E12.5 and analyzing embryos at E16.5. In the second part of this study, pregnant dams were dosed at E10.5, E12.5 and E17.5 and pups were analyzed at P14. As mentioned previously, due to a delay in the recombination event after Tamoxifen administration, labeled cells were those that expressed *Cux2* 12-24 hours after the time of administration. These experiments were conducted in order to determine the fate, and potential fate restriction, of *Cux2*-expressing progenitor populations during cerebellar development.

The cell fate analysis was performed by using well-described cell-specific markers to gauge the multipotency of the *Cux2*-expressing cerebellar progenitors. These markers included Calbindin, NeuN and Pax6. Calbindin is a known marker of cerebellar PCs, beginning at E14.5 (Wassef et al., 1985, Tolosa de Talamoni et al., 1993). Although *Cux2* was obviously expressed in the RL, we could not rule out the possibility that it may also be expressed in the VZ progenitors that give rise to PCs, glia and interneurons. If *Cux2* was being transcribed in PCs

then they would be labeled with the E10.5 dose and would be Calbindin-positive at the time of analysis since PCs are generated from E11-E12. The second marker that was selected was NeuN, a pan-neuronal marker extensively used to label cerebellar GCs (Mullen, Buck, Smith, 1992; Weyer and Schilling, 2003; Leung, Marino, 2004). At E16.5, NeuN is expressed in post-mitotic DCN neurons, as well as in a small population of post-mitotic GCs that are migrating inward (Fink et al., 2006, Chung et al., 2010). These two cell types each have distinct migratory paths and therefore can be distinguished qualitatively (Machold and Fishell, 2005, Fink et al., 2006, Chung et al., 2010). Furthermore, although NeuN marks post-mitotic neurons of the DCN, in the mature cerebellar cortex its expression is restricted to GCs. Specifically, it is not expressed in any molecularly identified interneuron population of the cortex, including UBCs (Weyer and Schilling, 2003). Therefore, at E16.5, NeuN is expressed in DCN neurons and in a small population of post-mitotic GCs and at P14 expression is faithfully restricted to DCN and GCs (Weyer and Schilling, 2003, Chung et al., 2010). The final marker that was chosen was Pax6, which begins expression in the RL between E11.5 and E12.5 (Fink et al., 2006, Chung et al., 2010). Pax6 is expressed in migrating DCN neurons beginning at E13.5 and is down-regulated as they enter the NTZ around this time (Fink et al., 2006). Therefore, Pax6 does not label DCN neurons at E16.5. In contrast, Pax6 is a consistent marker of GCs in the EGL and migrating post-mitotic, NeuN-positive GCs (Engelkamp et al., 1999, Chung et al., 2010). In summary, Calbindin identifies PCs at both stages of analyses, NeuN is predominantly in DCN neurons at E16.5 but in both DCN neurons and GCs at P14 and Pax6 is predominantly in GCs at E16.5.

4.4.1. *Cux2*+ Progenitors Give Rise to a Small Glial Population

When animals were dosed at E10.5 and analyzed at E16.5 *Cux2*-expressing cells gave rise to a small population of cells with radial glial morphology along the midline of the developing cerebellar anlage. These cells were in the rostral portion of the anlage, with oval-shaped cell bodies and processes that stretched down to the VZ and up to the pial surface. An immunohistochemistry experiment using GFAP, Nestin, Phospho-Vimentin and RC2 were all attempted, but labeled cells did not obviously co-label with any of these markers. These cells look similar to the RC2-expressing radial glia that serve as scaffolds for migrating PCs at E14.5; however, they are a much smaller population than previously reported for migration (Morales and Hatten, 2006). Therefore, these early *Cux2*-fated cells were anatomically consistent with a

radial glial progenitor lining the cerebellar ventricular region. The fact that very few cells were labeled likely reflected the normally low levels of *Cux2* expression at E10.5 (Iulianella et al, 2008).

4.4.2. *Cux2* is Fate Restricted to Granule Cells Throughout Development

An interesting finding from my study was the remarkably consistent GC labeling of the *Cux2-Cre* driven reporter labeling over the entire period of GC neurogenesis in the developing cerebellum. When animals were dosed at E10.5 and analyzed at E16.5 there was very little labeling, with no huge clusters of labeled cells. At E11-12 the cells of the DCN neurons are being generated from the RL, along with PCs from the VZ (Miale and Sidman, 1961, Goldowitz et al., 1997, Machold and Fishell, 2005, Fink et al., 2006, Morales and Hatten, 2006). Therefore, if *Cux2* was expressed in the progenitors of either of these cell types as they were generated then there would have been a significant amount of co-labeling with NeuN and Calbindin, respectively. However, there was a very low (if any) co-labeling with either of these factors. Furthermore, by E16.5 the DCN neurons are obvious as they are situated deep in the rostral half of the anlage in a clear nuclear cluster (Fink et al., 2006). If these neurons expressed *Cux2* when generated then there would have been a clear cluster of tomato-positive cells in these animals, but this was not observed. In fact, the majority of tomato-labeled cells in these animals co-expressed Pax6 (Engelkamp et al., 1999, Chung et al., 2010). Specifically, these cells were found in the EGL, the site of GC precursors during this stage of development. These findings were interesting since GCs are not yet being born at E11 and only begin being generated around E12.5 (Machold and Fishell, 2005). Clearly, the precursors of this population existed at E11-12, the time of dosing, and already expressed *Cux2*. Interestingly, when *Math1*^{CreERT2} animals are dosed with Tamoxifen at E10.5 and E11.5 there is abundant labeling of DCN neurons when analyzed at E12.5 and E14.5 (Machold and Fishell, 2005). When analyzed at E16.5, *Cux2*-expressing progenitors mostly become Pax6-positive GCs, in contrast to the DCN fate of *Math1*-expressing progenitors at the same time (Machold and Fishell, 2005}. Thus, while *Cux2* showed a similar pattern of expression as *Math1* in the developing cerebellar anlage, *Cux2* appears to fate label only a subset of neurons originating from this region, namely GCs. My findings therefore suggest that the *Cux2*-expressing cerebellar progenitors are highly restricted to a GC fate.

This fate restriction was maintained when animals were dosed at E10.5 and analyzed at P14, as tomato-positive cells predominantly co-expressed NeuN, a GC marker at this stage. However, there were a couple of PCs that were tomato-positive, but this was extremely rare. Overall, there was a very noticeable increase in the number of labeled cells compared to the animals analyzed at E16.5, suggesting a high proliferative capacity of the early cohort of *Cux2*-expressing cerebellar progenitors. In contrast, previous findings using the *Math1*^{CreERT2} mice dosed at E10.5 or E11.5 and analyzed at P21 showed no labeling of GCs (Machold and Fishell, 2005). These results, along with other studies, have led to the idea that *Math1* expression is renewed for each cell population that is generated from the RL (Machold and Fishell, 2005, Yeung et al., 2014). For example, the *Math1*^{CreERT2} animals dosed at E11.5 had no labeled cells still in the RL the following day, meaning that all of the cells that had expressed *Math1* the previous day had migrated out of this region. However, when animals were dosed at E12.5 there was significant labeling of GCs in the EGL and RL at E14.5, suggesting that *Math1* was still expressed in this region, but in a new cohort of progenitors. These results suggest that each RL population is generated from a new cluster of precursors that experiences a new wave of Math1 expression (Machold and Fishell, 2005, Yeung et al., 2014). This contrasts with *Cux2* fate-mapping studies reported here, which reflected a consistent expression of *Cux2* in GC-generating progenitors from their onset at E11.5-12.5 to the final proliferative exhaustion at perinatal stages.

It was interesting that DCN neurons were not labeled by the *Cux2*$^{CreERT2/+}$; *Rosa26*$^{tdTomato/tdTomato}$ genetic strategy when animals were dosed at E10.5, but GCs were labeled, in contrast to prevailing view that GCs are not yet being generated in the RL primordium at E10.5 (Machold and Fishell, 2005). It is possible that the GC precursor population was actually beginning to be generated at this point, but did not yet express *Math*1. In this case, my findings have identified an earlier cohort of GC progenitors that were specifically labeled by *Cux2* genetic recombination strategy. Importantly, this finding extends the timeline of the onset of GC neurogenesis by 1 day earlier than previously thought. In summary, although the *Cux2* levels were low in the cerebellar primodium at E11, the fate mapping of this domain predominantly restricted to GC precursors over PCs and DCN neurons, highlighting a remarkably specific fate preference of the *Cux2*-expressing cerebellar progenitors.

There was a large difference in the amount of labeling between animals dosed with Tamoxifen at E10.5 relative to dosing at E12.5. When animals were dosed with Tamoxifen at

E12.5 and analyzed at E16.5 there was abundant labeling in the RL and EGL, in line with the observed *Cux2* mRNA expression in the RL at E13.5. The drastic increase in labeling was consistent with the restriction of *Cux2*-expressing progenitors to a GC fate (Fig. 8). For example, at E11-12 GCs are not yet being born and there was little labeling, but at E13.5-14.5 GC generation is abundant and there was a lot more *Cux2-Cre* driven tomato-labeling. The labeling pattern was similar to results reported from $Math2^{CreERT2}$ animals dosed at E12.5 (Machold and Fishell, 2005). In addition, at E16.5, GCs begin to express NeuN as they migrate deeper to form the IGL (Chung et al., 2010), accounting for the increased co-expression of NeuN in tomato-positive cells observed at E12.5 compared with E10.5 dosing of the $Cux2^{CreERT2/+}$:$Rosa26^{tdTomato/tdTomato}$ mice. However, we cannot exclude the possibility that some of the NeuN-positive cells were DCN neurons, and not maturing GCs. Even if *Cux2* fate mapped to DCN neurons, it would still be a very small percentage of the population, since by my calculation the DCN labeling was less than 17% of the total number of tomato-positive cells. Furthermore, Pax6 is down-regulated in DCN neurons beginning before E16.5, meaning that all Pax6-positive cells in the EGL were, in fact, GCs (Fink et al., 2006). The dosing of the $Cux2^{CreERT2}$/$Rosa26^{tdTomato/tdTomato}$ mice at either E12.5 or E17.5, when GCs are being generated and are actively proliferating, resulted in extensive labeling of the GCL at P14. The labeled cells in both of these cohorts were almost exclusively co-labeled with NeuN, a marker of GCs in the GCL (Weyer and Schilling, 2003). Therefore all of these results continued to highlight the remarkably specific pattern of GC fate restriction of *Cux2*-expressing progenitors during cerebellar development.

4.4.3. Cux2 Progenitors in the Rhombic Lip

The restriction of *Cux2* to GC precursors was very interesting in light of recent results that suggest a potential compartmentalization of the RL. It has been proposed that RL cells move linearly from the internal to external compartments before migrating up into the EGL (Yeung et al., 2014). These studies prompted the search for additional factors involved in restricting RL progenitors to a particular fate. The *Cux2*-driven tomato-positive cells we observed did not seem to be confined to a single region, but were present uniformly throughout the RL (Fig. 6). However, these results did not necessarily contradict the previous view of a compartmentalized RL. For example, by analyzing the fate-mapped animals at E16.5, we could not infer current

Cux2 expression patterns, but rather only describe the phenotypic fate of cells that once expressed *Cux2* at E13-14. It was possible that *Cux2* was only expressed in a single RL compartment and that this cluster of cells moved laterally between compartments while down-regulating *Cux2* locus activity. If *Cux2* expression was restricted to cells within the interior portion of the RL, then the resulting daughter cells would move to the exterior RL and begin expressing *Math1* around E12-13 once GC generation began. This interpretation is in line with our observation that *Cux2* was expressed in GC precursors prior to expressing *Math1* (Machold and Fishell, 2005). Furthermore, since protein did not seem to be expressed in the cerebellum until E16.5, or at least was not detected by our antibody, our immunofluorescence analysis could not help elucidate whether or not *Cux2* was restricted to expression in a single compartment. Therefore, these results suggested that the progenitors that expressed *Cux2* at E13.5-14.5 did not remain confined in a single RL compartment, but dispersed to generate GC precursors that proliferated and extensively populated the GCL in the fully formed cerebellum.

4.4.4. Variability in Cux2 Labeling

Another interesting result that seemed consistent between mRNA expression and fate-mapping patterns was the striped, or domain-restricted, expression of *Cux2* in the developing cerebellum. For example, when animals were dosed at E12.5 or E17.5 there was a large range of the extent of labeling along the mediolateral axis, with some axial levels having no labeling and others having all folia labeled, suggesting potential parasagittal domains defined by restricted *Cux2* labeling. These results were consistent with the striped *Cux2* mRNA expression pattern observed in the chick at E10 (HH stage 36) and E11 (HH stage 37) that was reminiscent of patterns of genes involved in establishing functional cerebellar network units. Differential molecular expression patterns have been previously reported within GC compartments along the entire cerebellum that are thought to reflect the patterning of these cells into functional units (Frantz et al., 1994, Hawkes and Turner, 1994, Alam et al., 1996, Ozol and Hawkes, 1997, Rogers et al., 1999). This phenomenon is similar to the restricted expression of markers during cortical neurogenesis that results in 6 separate layers of cells, each involved in distinct functions. Interestingly, Cux2 has been hypothesized to restrict cortical SVZ progenitors to adopt an upper layer pyramidal cell fate, which have a distinct projection pattern and function from other cortical projection neurons, although this finding has been recently debated in the literature

(Franco et al., 2012, Guo et al., 2013, Eckler et al., 2015). Similarly, if *Cux2* expression is restricted to GCs, then it could possibly play a role in defining functional subsets of these cells. Specifically, it is possible that *Cux2* expression is restricted to distinct parasagittal domains in the GCL, playing a role in helping subpopulations of GCs establish mature characteristics required for their functional units (Consalez and Hawkes, 2012). Interestingly, fate map studies using the *Math1*^{CreERT2} animals did not report any significant mediolateral variability in labeling, reflecting another crucial difference with the GC-specific *Cux2*^{CreERT2} labeling strategy described here (Machold and Fishell, 2005). At the least it argues for the use of different GC-specific Cre drivers in cerebellar fate mapping studies. However, it points to an intriguing possibility that not all GC precursors are equivalent in terms of their final phenoytypic maturation. There also remains the possibility that the *Math1*^{CreERT2} driver does indeed differentially label GC subdomains but that this observation has yet to be reported. If this is the case, then my fate mapping of the *Cux2*^{CreERT} line simply reflects the developmental progression of GC generation and maturation.

In addition to a large amount of mediolateral variability, our results also revealed a strong difference in anterior vs. posterior labeling depending on the dosing time point. For example, when *Cux2*^{CreERT2} animals were dosed with Tamoxifen at E12.5 there was abundant labeling in the posterior folia at P14, with minimal to no labeling in anterior folia. In contrast when animals were dosed at E17.5 there was significantly more labeling in the anterior folia. In both of these cohorts some mediolateral axial levels had labeling only in the most anterior or posterior folia, while other levels had labeling in all folia with a strong anterior or posterior gradient (Fig. 10). Interestingly, these results seem to be the opposite from what has been reported for the *Math1*^{CreERT2} mice. When Math1 animals are dosed at E12.5 there is much stronger labeling of anterior folia at P21 and little or no labeling of posterior folia. In addition, when these animals are dosed at E16.5, there is abundant labeling throughout all of the folia at P21 (Machold and Fishell, 2005). The results from Math1 animals support previous results that suggest that GCs develop in an anterior to posterior manner (Wingate and Hatten, 1999, Machold and Fishell, 2005). In contrast, *Cux2* fate mapping seemed to show the reverse pattern of labeling. It is important to frame this finding relative to when and where *Cux2* expression initiates in the GC precursors. *Cux2* did not seem to be expressed in cells as they were actively migrating away from the RL at E13.5-14.5. It is at this stage when GC progenitors populating the anterior folia

are generated. It is therefore not surprising why I observed minimal tomato labeling in anterior folia resulting from the *Cux2*^{CreERT2} fate mapping at E13.4-14.5. Furthermore, at E18.5-19.5 when the last of the posterior GC precursors are leaving the RL, they again did not express *Cux2*, since Tamoxifen dosing of our *Cux2* reporter mice at this stage predominantly labeled anterior folia. This raises the possibility that *Cux2* functions in patterning the RL and EGL resulting in their molecular segmentation along a rostro-caudal gradient, rather than in directing early migration of GC.

4.5. Proposed Mechanism of *Cux2* in Cerebellar Development

This study was able to elucidate that *Cux2* was strongly expressed in the cerebellum during development and revealed a surprising specificity to GC identity and GCL regionalization. Based on previous work, I hypothesize that *Cux2* functions as part of the Notch signaling pathway to coordinate GC differentiation from RL progenitors during cerebellar development (Iulianella et al., 2009, Wittmann et al., 2014). Notch signaling is important for regulating proliferation and differentiation during neurogenesis by lateral inhibition (Artavanis-Tsakonas et al., 1995, Lewis, 1996). Notch signaling acts by regulating the expression of a particular class of transcription factors belonging to the bHLH family, of which Math1 is a member (Kageyama and Nakanishi, 1997). These factors regulate the timing of neuronal differentiation and maturation by suppressing the transcription of pro-neural genes (Kageyama and Nakanishi, 1997, Kageyama et al., 2008). For example, Math1 is important in regulating proliferation of neuronal precursors, while NeuroD is important in terminal differentiation, both are involved in GC development and are sensitive to Notch signaling (Kageyama and Nakanishi, 1997, Machold et al., 2007). Therefore, it is a likely that *Cux2* functions as part of this signaling pathway in the cerebellum, feeding into the regulation of bHLH factors, as was observed previously in the spinal cord (Iulianella et at, 2009).

Notch signaling has been shown to be very important for the proper development of GCs in the cerebellum (Kageyama and Nakanishi, 1997, Solecki et al., 2001, Gazit et al., 2004). Throughout cerebellar development Notch has been shown to be important in the RL in promoting the transition of progenitors to generate a new neuronal population. In addition, it is important to maintain a proliferative population to promote subsequent cell fate transitions by controlling responsiveness of cells in this region to neurogenic inductive signaling from the RP,

encouraging cells to remain in a proliferative state (Machold et al., 2007). For example, Notch2 is expressed in proliferating GCs before they begin differentiation and inward migration, and promotes a proliferative phenotype (Solecki et al., 2001). The importance of proper signaling in normal development is evident in the implication of Notch2 over-activation in GC over-proliferation and human medulloblastomas (Xu et al., 2009). Therefore, clearly Notch signaling is important during GC development in maintaining GCs in a proliferative state.

It has been suggested that Math1 regulates differentiation at least in part through its feedback on components of the Notch signaling pathway (Gazit et al., 2004). *Math1*-null mutants do not show early signs of proper GC differentiation and fail to extend neurites, highlighting its role in GC maturation. In addition, at E14.5 Notch signaling receptors in the RL are down-regulated when *Math1* is knocked out (Gazit et al., 2004). In contrast, when *Notch1* is knocked out in the developing cerebellum, there is an increase of *Math1* expression in the RL. By E12.5, these animals have a drastic reduction in cerebellar size, with a lasting increase in *Math1* expression. These experiments suggest that *Math1* can regulate Notch signaling while this pathway can in-turn negatively regulate *Math1* (Machold et al., 2007). Furthermore, *Math1* feedback loops have been shown to be important in the transitions in the generation of different cell types from the RL. For example, experiments suggest that *Math1* expression is renewed in the precursors of each neuronal population generated from the RL. Specifically, *Math1* is down-regulated at the end of the generation of brainstem nuclei and then again at the end of DCN neuron generation. In addition, the *Math1*-positive cells that give rise to the DCN neurons are not the same cells that give rise to the GC. These results highlight the complex feedback mechanisms that are involved in orchestrating the balance between progenitor proliferation and differentiation in the developing cerebellum (Gazit et al., 2004, Machold and Fishell, 2005, Yeung et al., 2014).

Previous studies suggest that Cux2 is tightly linked to Notch signaling. Initially, *cut* was identified in *Drosophila* as an effector of Notch signaling during wing development (Micchelli et al., 1997). In the spinal cord, Notch signaling maintains precursors in an undifferentiated proliferative state, inhibiting the activation of neurogenic factors (Kageyama et al., 2007, Iulianella et al., 2009). Our lab has previously reported that Cux2 genetically acts downstream of Notch signaling in the developing spinal cord (Iulianella et al., 2009). Furthermore, we recently showed that in the olfactory epithelium either *Cux2* over-expression or knock-down negatively regulates Notch signaling, resulting in a decrease in both proliferation and neurogenesis

(Wittmann et al., 2014). These results highlight the important role of maintaining a balance of Cux2 to prime Notch signaling in order to keep progenitors in a proliferative state while at the same time driving neurogenesis. Therefore, since Cux2 has been shown to act downstream of Notch and regulate the expression of at least a subset of bHLH proteins (Kageyama and Nakanishi, 1997, Iulianella et al., 2009), I suggest that *Cux2* acts to regulate the extent of Notch signaling in the RL to limit the multipotency of that population and regulate the timed neurogenesis of GCs.

The mechanism by which Notch and Math1 regulate each other is not understood. It is possible that *Cux2* functions in Notch signaling and helps regulate the development of GCs, perhaps by regulating Math1 expression. Since Notch signaling positively regulates Cux2, which in turn restricts Notch signaling, this feedback control may also be a mechanism to ensure the timed generation of GCs (Wittmann et al., 2014). This hypothesis is also supported by previous studies in the spinal cord in which Notch was shown to positively regulate Cux2, which then positively regulates another HLH gene (Iulianella et al., 2009). Furthermore, since Notch can negatively regulate Math1 expression (Machold et al., 2007), the Notch1-Cux2 regulator loop may blunt the inhibition of Notch on Math1, resulting in the induction of *Math1* in the RL (Fig. 13). This model is consistent with our observation that *Cux2* expression does in fact precede *Math1* in the RL, and *Cux2* locus activity is associated with the restriction of subset of progenitors to GCs. Furthermore, the complimentary expression pattern of *Math1* and *Cux2* strengthens the possibility of complex feedback regulatory loops involving Notch during GC development. For instance, in $Math1^{CreERT2}$ mice, dosing at E10.5 labels all of the DCN neurons, while dosing $Cux2^{CreERT2}$ animals at the same time does label DCN neurons, but instead restricts to GC precursors. This reveals that *Cux2* is expressed in GC progenitors prior to the acquisition of *Math1* expression, potentially helping to promote a GC fate following DCN neuron generation by activating or stabilizing *Math1* expression. Since *Cux2* is restricted to GCs in the cerebellum, it could possibly identify a subset of neural progenitors in the RL destined to become GCs by feeding into the *Notch-Math1* loop to bypass the formation of other *Math1*-expressing neuron subtypes, such as DCN neurons.

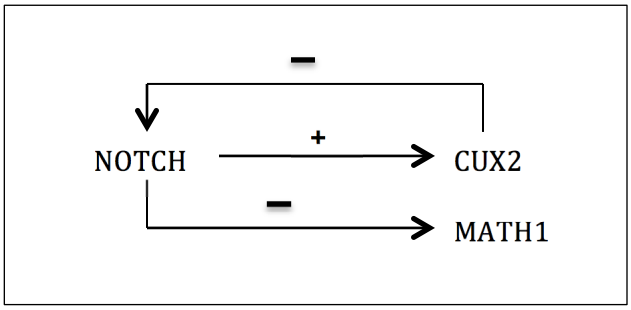

Figure 13. This is a simple model of the proposed mechanism by which Cux2 exerts its function during GC development. Cux2 may provide dis-inhibition of Math1, by inhibiting Notch signaling, to promote its expression in the RL and in the newly generated GC progenitors. This may provide insight into the mechanisms that control Math1 expression renewal in new progenitors in the RL.

4.6. *Cux2* Gain-of-Function Cannot Change the Fate of Progenitors

The tomato fate mapping results highlighted the restriction of *Cux2*-expressing cells to give rise solely to GCs. When *Cux2* was overexpressed in the developing chick or mouse spinal cord it resulted in an increase in early differentiation markers and revealed an important role of *Cux2* in neural specification, maturation and migration (Iulianella et al., 2008). Furthermore, *Cux2* hypomorphs have a decrease in spinal cord interneuron populations, highlighting the importance of Cux2 in the proliferation and specification of this subpopulation (Iulianella et al., 2009). All of these results highlight the important role of Cux2 in early cell fate specification. Therefore, based on the restricted labeling of *Cux2*-expressing progenitors to GCs, I tested the ability of constitutive high levels of *Cux2* to bias the fate in the chicken embryonic hindbrain region towards a GC fate. Using a bicistronic vector, *Cux2* and GFP were overexpressed in chick dorsal neuropepithelium at E2 (HH 11-13), when hindbrain is being patterned, and the resulting embryos were subsequently analyzed at E9 (HH 35), corresponding to the stage when GCs have begun their inward migration from the EGL. However, embryos over-expressing *Cux2* showed no biases in the formation of the major cerebellar derivatives of the hindbrain when compared to the GFP electroporated controls. Specifically, *Cux2*-overexpressing and GFP control cells displayed the same probability of co-labeling with Pax6 (GC precursors), Calbindin (PCs) or NeuN (DCN neurons and GCs). These results highlighted that *Cux2* by itself is insufficient to influence the specification of GCs from a precursor pool since it could not increase the proportion of GCs produced. However, this does not negate the hypothesis that *Cux2* acts in the Notch signaling pathway with *Math1*.

Superficially these results seem at odds with the distinctly fate restricted behaviour of *Cux2* activity. Yet this observation is supported by the fact that *Cux2* is potentially not expressed in the entire GC population, suggesting instead that it may play a role in the regionalization of GCs. Another possible explanation of the lack of significant results may be due to necessarily early timing of the electroporation experiments. GC neurogenesis begins in the chick around E6 (HH 28-30), but in our study chicken embryos were electroporated at E2 (HH 11-13) (Ryder and Cepko, 1994). This early time point for electroporation was chosen simply because of the technical difficulty of electroporating the chicken neuroepithelium after E3 (HH 18-22), when vascularization of the embryo is extensive and sensitive to the cauterizing effect of the electroporation methodology. In addition, we wanted to choose a stage that would be before

Math1 expression to test for the sufficiency of *Cux2* as a GC determinant. In retrospect, this was an illuminating experiment, since it suggests that the role of *Cux2* in GC development may be dependent on Math1 activity occurring concomitantly in RL progenitors. This leads one to predict that if *Cux2* was overexpressed at a later stage, particular if co-expressed with *Math1*, it may promote the acquisition of the GC fate. A model summarizing this hypothesis is depicted in Figure 14. Another possibility is that Cux2 and Cux1 have over-lapping functions in the developing cerebellum, resulting in a recovery by Cux1 when Cux2 activity is perturbed, as in overexpression studies. This possibility is supported by the strong expression of Cux1 in the developing cerebellar primordium at E12.5. However, this possibility is unlikely since an overexpression of Cux2 would result in an over-activation of the entire Cux1/Cux2 functional system if they are, in fact, redundant. Therefore, the most likely explanations are that either Cux2 is not itself necessary for fate determination, or electroporation experiments were performed too early to affect the *in vivo* function of Cux2.

4.7. Implications for Future Work

The present study was able to identify a role for *Cux2* in GC fate in the developing cerebellum. However, many of the results highlight the complexity of cell fate restriction in a collection of neural progenitors that prompts further investigation.

Further experiments should be aimed at understanding the significance of the striped *Cux2* expression in the chicken cerebellum anlage and whether it is related to the variation in mediolateral labeling observed in the $Cux2^{CreERT2}$ fate mapping analysis. The discrete striped *Cux2* mRNA expression in the developing chick cerebellum is very intriguing in light of the fact that there appears to be molecular coding in the cerebellar anlage that may have profound influence in organizing the cerebellum into functional units. A comparison of *Cux2* expression with other striped expression markers, such as *En2* or *BMP7*, would reveal whether it is co-expressed in the same pre-set domains or whether it defines a novel functional domain altogether. Beyond expression pattern, retrograde tracing of the incoming mossy fibers would reveal whether these domains group into distinct functional anatomical units associated with particular cerebellar functions. For example, if incoming fibers from a specific pre-cerebellar nucleus preferentially synapses on cells that are derived from the descendants of *Cux2*-fate

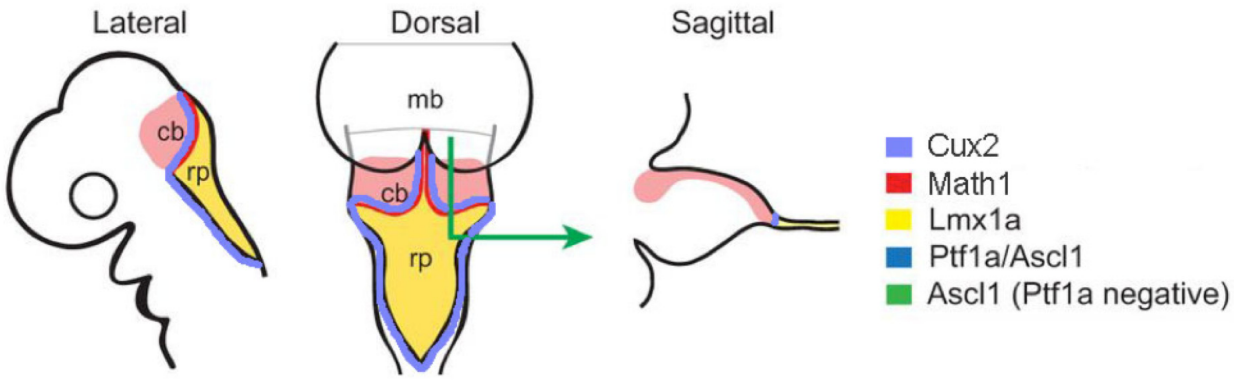

Figure 14. A model revealing the expression pattern of Cux2 compared to Math1 in the developing cerebellum. Cux2 is expressed throughout the RL, with a similar pattern to Math1 in the rostral RL during the generation of GC at E13.5. The Math1-expressing domain gives rise to all RL derivatives, in contrast the Cux2-expressing domains seems to only give rise to GC progenitors, highlighting it as a novel marker of strict GC progenitors. Adapted with permission (Butts et al., 2014).

mapped progenitors then this will provide evidence for the early molecular segregation of functional distinction populations of cells within the GCL.

At a more technical level, to address the significant variation observed in the *Cux2* fate mapping data, future studies should use multiple doses of Tamoxifen throughout the entire period of GC generation, namely daily from E12.5 through to E17.5, to determine if the regionalized mediolateral and rostrocaudal GCL labeling is maintained. If so, that would confirm that *Cux2* activity marks a subset of GCs in the cerebellum that may have consequences for the development of functional neural networks. This type of experiment will shed light on whether *Cux2* is actually expressed in all GC progenitors throughout the cerebellum, undergoing dynamic changes in its expression that reflects the patterned genesis of GCs from the posterior to anterior and in the mediolateral direction.

An interesting observation noted in this work concerns the disparity between Cux2 mRNA and protein expression. Specifically, although it has been previously reported that Cux2 protein may not always be expressed in cells that make the mRNA, it should be further investigated in the developing cerebellum. The reason for this post-transcriptional regulation is entirely unknown but may relate to technical issues, such as the lack of sensitivity of our Cux2 antibody. However, more interestingly it may reflect that there may be different Cux2 isoforms not recognized by our carboxyl-terminal Cux2 antibody. This question can be addressed by testing Cux2 antibodies specific to different amino-terminal regions of the protein. Yet another possibility is that the developing RL region expresses a protein that inhibits *Cux2* translation, or promotes its degradation, in order to carefully control its levels at a time when progenitors are sensitive to *Cux2*-mediated neurogenesis. Other protein extraction methods would be able to confirm the absence of Cux2 protein in this region, paving the way for further investigation into potentially important post-transcriptional regulation of this gene. Understanding the distinct expression patterns of both the protein and mRNA will elucidate possible differences in function between the two gene forms.

Finally, the potential role of *Cux2* in Notch signaling in the developing cerebellum should be fully elucidated. In light of this it is important to re-iterate that Notch signaling and Math1 expression are tightly linked during cerebellar development, but the exact mechanisms that help regulate these factors are not completely understood. Based on previous observations in the spinal cord and olfactory epithelium, the ability of *Cux2* to negatively regulate Notch signaling

in cerebellar progenitors should be investigated. Our results reveal that at E11.5-12.5 GC precursors already express *Cux2*, although they have yet to express Math1 and begin their migration from RL. Thus it is possible that *Cux2* acts in the RL or EGL to restrict Notch signaling in part by regulating Math1, and in turn be regulated by Math1, forming a mutually reinforcing regulatory loop to lock in GC fate. Future studies should aim to investigate these interactions for both *Cux2* and *Math1*. Furthermore, it should be determined if *Cux2* mouse mutants have a disturbance in the initiation or maintenance of Math1 in the RL progenitors, and the subsequent effect on GC number in post-natal life.

4.8. Conclusion

The present study provides evidence that *Cux2* activity was remarkably restricted to regions involved in GC development, namely the RL and EGL. Furthermore, we showed that *Cux2* progenitors were fate restricted throughout a broad window of development to become mature GCs. We also showed that *Cux2*-expressing progenitors displayed a different developmental pattern relative to *Math1*-expressing cells, suggesting that they could both be used to study the regionalization of the GCL during development. This opens up a novel area of study in cerebellar morphogenesis with the potential to uncover how functional domains are set up. Finally, we show that *Cux2*, on its own, was unable to cause fate changes in very early hindbrain progenitor populations. Overall, my work revealed that *Cux2* activity within the RL patterns a GC fate during cerebellar development. This in turn may have functional consequences in the formation of anatomically distinct cerebellar networks, and lays the foundation for further studies in the formation of the intriguing structure referred to by Cajal as the “little brain”.

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Appendix

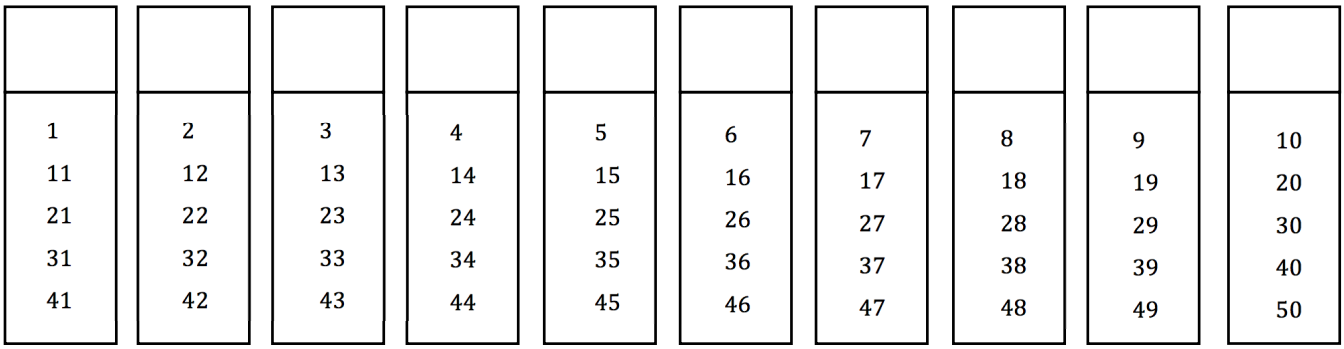

Figure Ai. Example of the serial slide sections used in each portion of the study. Numbers on the slides refer to the section number. Serial sectioning ensures that each slide contains sections from multiple axial levels and avoids necessarily staining all slides.

Restriction Digest	
cCux2 Plasmid (µg)	5
Not1 Enzyme (µL)	1
Buffer 3 (µL)	2
H_20 (µL)	10
10x BSA (µL)	2
Total Volume (µL)	20

Table 1. Reagents used in the restriction digest in the preparation of riboprobes for ISH experiments.

Riboprobe Synthesis	
1µg/µL Linear DNA Template (µL)	1
DEPC-treated, autoclaved H_20 (µL)	8
5x Transcription Buffer (µL)	4
10x DIG Labeling Mix (µL)	2
10mM DTT (µL)	2
Placental RNase Inhibitor (µL)	1.5
RNA Polymerase T3 (µL)	1.5
Total Volume (µL)	20

Table 2. Reagents used to synthesize the riboprobes for ISH experiments.

Hybridization Buffer	
Formamide (mL)	25
20x SSC (mL)	12.5
Yeast torula-RNA (mg)	50
Dextran Sulfate (50%; mL)	5
Denhardt's (50x;mL)	1
DEPC-treated H_2O	Up to 50 mL
Total Volume (mL)	50

Table 3. Reagents used to prepare the hybridization buffer for ISH experiments.

TBST	
1M Tris/HCl pH=7.5 (mL)	2.5
1M NaCl (mL)	7.5
1M KCl (mL)	0.5
Triton X-100 (mL)	0.5
DEPC-treated H_2O (mL)	39
Total Volume (mL)	50

Table 4. Reagents used to prepare TBST for ISH experiments.

Alkaline Phosphatase Buffer (NTMT)	
Purchased molecular biograde H_2O (mL)	3.75
1M Tris/HCl pH=9.5 (mL)	0.5
1M NaCl (mL)	0.5
1M $MgCl_2$ (mL)	0.25
Tween20 (µL)	5
Total Volume (mL)	5

Table 5. Reagents used to prepare NTMT for ISH experiments.

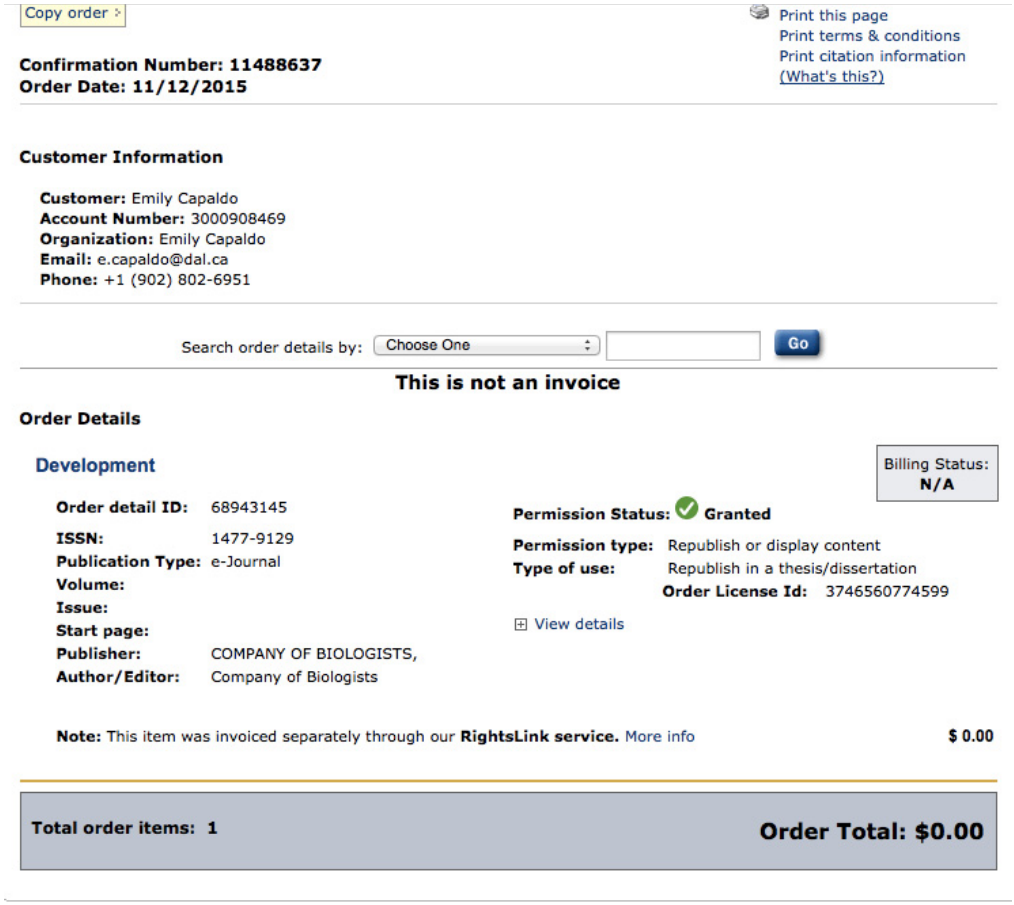

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Figure Aii. Access granted to reproduce Figures 1 and 14 in my written thesis. The come from the same figure in a paper published in Development.