FEZ FAMILY TRANSCRIPTION FACTORS DURING NERVOUS SYSTEM DEVELOPMENT

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Table S1 available online
Abstract

Fez Family Transcription Factors During Nervous System Development

Matthew J. Eckler

The profound diversity of cell types within the nervous system has been appreciated for greater than a century. However, the mechanisms by which these different cell types are generated during development remain elusive. Here I focus on the roles of two closely related zinc-finger transcription factors, \textit{Fezf1} and \textit{Fezf2}, during development of the olfactory system and cerebral cortex. I show that \textit{Fezf1} functions to promote the development and cell fate specification of sensory neurons within the main olfactory epithelium. Conversely, \textit{Fezf2} is required for developmental maintenance of the vomeronasal organ. Further, I show that during development of the cerebral cortex, \textit{Fezf2} expression identifies a multipotent progenitor for neocortical projection neurons and glia. Finally, I demonstrate that transcription of \textit{Fezf2} is regulated by multiple non-coding DNA elements that promote its expression across distinct domains of the cerebral cortex. Collectively, these studies illustrate the critical functions of Fez family transcription factors during development of the mammalian nervous system.
Acknowledgements

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Finally, this work was the effort of many. The text of this dissertation contains work that was previously published or is in submission for publication:


Chapter 1: Introduction

Our nervous system underlies our perceptions, thoughts and behaviors (Breed and Sanchez, 2012). In accordance with its fundamental role in the human experience, defects in nervous system function manifest as devastating disorders such as mental retardation, autism and schizophrenia. These disorders all have some developmental origin and thus highlight the importance of understanding neuronal development in order to address their underlying etiology (Daily et al., 2000; DiCicco-Bloom et al., 2006; Rodier et al., 1996; Weinberger and Levitt, 2011).

The diversity of cell types within the human nervous system is immense. The human brain alone contains approximately 100 billion neurons, of which there are at least 1000 different subtypes distinguished by their differing morphology, connections and molecular expression profiles (Kandel et al., 2000). Since the work of Ramon y Cajal and Camilllio Golgi more than a century ago, biologists have strived to catalogue the different cell types in the nervous system. However, lagging behind these efforts is an understanding of the mechanisms that generate cellular diversity during its development.

Nervous system development

Development of the vertebrate nervous system includes the differentiation of immature neurons and glia from stem cell precursors, the migration of these immature cells from the stem cell niche to their final locations, the extension of axons and
dendrites and ultimately synapse formation contributing to the establishment of neural circuits. This process is controlled through secreted signaling molecules, transcriptional regulators and cellular adhesion and recognition molecules that ultimately function to determine a cell’s identity, position and connections within the nervous system. The initial circuits that are formed through this process are then refined during critical periods of postnatal development through experience-dependent changes in gene expression that effect neuronal growth and synapse formation. Many of the same mechanisms that help to generate the nervous system during embryogenesis are retained into adulthood and help to maintain normal brain function as well as changes in circuitry stemming from life experiences. Further, some of these developmental mechanisms are also disrupted in late-onset diseases such as Parkinsons and Alzheimers raising the possibility that modifying or repairing these cellular processes may help to alleviate symptoms of these devastating conditions (Arendt, 2001; Rodier et al., 1996; Shadrina et al., 2010).

**Nervous system induction**

The developing nervous system is first discernable early in gestation as a thickening of the ectodermal cell layer (Gilbert, 2010). Termed the neural plate, this structure is located opposite of the primitive streak that will in contrast give rise to the notochord (Sanes et al., 2012). Following its induction, the neural plate folds inward along the midline to generate the neural tube that contains stem cells that will give rise to the central nervous system (CNS) (Rao and Jacobson, 2005). Cells located
along the ventral midline of the neural tube differentiate into a thin strip of epithelial-like cells that form the floorplate (Wolpert, 2011). This structure secretes signaling molecules that help to specify the identity and lineage potential of adjacent neuroblast cells that will give rise to ventral brain structures (Yamada et al., 1991). Similarly, a narrow strip of neuroepithelium along the dorsal midline generates the roofplate that secretes molecular signals to pattern dorsal CNS structures (Chizhikov and Millen, 2004). Cells at the lateral edges of the neural fold generate the neural crest. Neural crest cells migrate away from the neural tube and reside along its dorsal edges where they will eventually give rise to the peripheral nervous system (PNS) as well as cartilage and bone of the face and skull (Gilbert, 2010).

Seminal transplantation experiments in *Xenopus* oocytes during the early 20th century by Hans Spemann and Hilde Mangold demonstrated that the neural plate is initially induced from uncommitted ectodermal tissue in response to signals from adjacent mesoderm (Spemann, 1924). Termed the Spemann Organizer, this structure was shown to secrete factors that promote neural plate formation. Later identified to include follistatin, noggin and chordin, these factors pattern the neural plate through inhibition of BMP signaling (Hemmati-Brivanlou et al., 1994; Lamb et al., 1993; Piccolo et al., 1996). Additional studies have demonstrated that neuroectoderm is the default fate of ectodermal tissue during development and thus nervous system induction is a permissive process (Hemmati-Brivanlou and Melton, 1994).

**Patterning of the nervous system**
Following neural induction, the developing nervous system is patterned through a combination of secreted molecules that function to differentiate the neural tube into different regions of the CNS. Through this process, the distinct lineage potential of neural precursors are specified across spatial domains along the anterior-posterior and medial-lateral axis of the neural tube. Just as neural ectoderm is the default ectodermal fate, an anterior fate is the default fate of the neural tube. Signaling induced by secreted molecules such as retinoic acid (RA) and Wnt function to promote a posterior fate in the caudal neural tube. (Blumberg et al., 1997; Yamaguchi, 2001).

Secreted molecules from adjacent tissues similarly control the dorsal-ventral patterning of the neural tube. The roof plate secretes BMPs while the floor plate and notocord produce Sonic Hedgehog (Shh) (Jessell and Sanes, 2000). Within the anterior neural tube these signals function to promote the dorsal versus ventral identity of forebrain precursors (Rowitch and Kriegstein, 2010). Signals from the roof plate guide the generation of dorsal structures such as the cerebral cortex by inhibiting more ventral structures such as the ganglionic eminences (Lee and Jessell, 1999). The posterior regions of the neural tube give rise to the hindbrain and spinal cord. Patterning of these tissues is controlled by secreted factors from the roof and floor plate. Ventral regions exposed to high levels of Shh generate motor neurons while high levels of BMP signaling specify sensory input neurons (Jessell and Sanes, 2000). The combined activities of these signaling pathways along with others such as Wnts and retinoic acid coordinate the anterior-posterior and medial-lateral patterning.
of the neural tube, resulting in the precise control of downstream gene-expression programs. Ultimately this functions to promote the identity and differentiation potential of neural progenitor cells in a spatially restricted fashion.

Developmental patterning of the PNS is similar to that of the CNS. Neural crest cells are patterned though a combination of signals from the adjacent neural plate and ectoderm as well as the underlying mesoderm. Intermediate levels of BMP signaling and FGFs as well as RA and Wnt play pivotal roles in patterning the PNS from neural crest cells. Additionally, RA has been shown to be critical for olfactory system development. In mice with disrupted RA signaling, neural crest cells fail to migrate to the olfactory placode, resulting in severe defects in olfactory epithelium development (Anchan et al., 1997; LaMantia et al., 1993)

Mechanisms of cell fate specification during nervous system development

Significant progress has been made towards understanding the initial events that induce and pattern the nervous system. However, much less is known about the mechanisms that specify individual cell fates during development. Studies from model organisms indicate that the both genetics and environment are important for determining a cell’s fate during neural development.

Perhaps one of the best-studied examples is that of neuronal specification in the rodent spinal cord. Collectively, these studies indicate that the dorsal-ventral identity of neurons is initially specified by graded expression of the morphagens Shh and BMPs. Spatially related but distinct neuron subtypes are then refined by the
combinatorial expression of cell-type specific transcription factors. Ultimately, this has lead to the model of a transcription factor “code” that determines neural subtype identity (Jessell, 2000).

Neurons along the dorsal-ventral axis of the spinal cord can be grouped into five major classes. These are v0, v1, and v2 interneurons, motor neurons (MNs) and v3 interneurons respectively. Each of these cells types is generated from a distinct progenitor domain (Jessell, 2000). In the ventral neural tube, epithelial cells in the floor plate secrete the morphagen Shh as well as BMP inhibitors such as chordin and noggin. Alternatively, cells in the roof plate secrete high levels of BMP. The opposing activity of the roof plate and floor plate establish diametrically opposed gradients of Shh and Bmp signaling that result in the expression of cell type specific transcription factors. These factors ultimately specify progenitors cells with distinct lineage potentials along the dorso-ventral axis (Lupo et al., 2006). Progenitor cells closest to the floor plate receive high-levels of Shh that induces the expression of the homeobox transcription factors Nkx2.2 and Nkx6.1. Progenitors that express these factors give rise to the most ventral (v3) class of spinal cord interneurons. Alternatively, motor neuron precursors are located dorsal to v3 interneuron precursors and receive lower-levels of Shh that induces expression of the transcription factor Pax6. In a similar fashion, more dorsal interneurons classes (v2-v0) are exposed to progressively decreasing levels of Shh and increasing levels of Bmp that induce the expression of transcription factors such as Dbx2, Dbx1 and Pax7 respectively (Jessell, 2000).
The differential expression of transcription factors along the doral-vental axis of the neural tube not only marks distinct class of spinal cord precursors but also plays a critical role in the proper fate specification of these neuronal subtypes. For example, loss of Nkx2.2 expression in v3 interneuron precursors leads to the mis-specification of these cells and an expansion of the more dorsal MN population (Briscoe et al., 1999). Control over the generation of neurons versus glia is regulated in part by expression of the transcription factor Olig2. Olig2 has been shown to maintain MN precursors in an undifferentiated state to promote the generation of oligodendrocytes through repression of Ngn2, which instead directs the formation of MNs (Lee et al., 2005). Further subtype specification of MNs occurs thorough the combinatorial expression of LIM domain transcription factors where specific subtypes of motor neurons are specified though the expression of Islet1, Islet2, Lim1 and Lim2 (Tsuchida et al., 1994). Collectively, these studies demonstrate how the combinatorial expression of a transcription factor “code” is established in response to differing levels of secreted signaling molecules. Selective expression of cell type specific transcription factors in response to signaling gradients promote proper neuronal subtype as well as inhibit neighboring subtypes through lateral inhibition.

Similarly, in the fruit fly Drosophila Melanogaster, neuronal subtype is specified by the selected temporal expression of different transcription factors as neuroblast cells undergo asymmetric divisions to generate neurons. During development, neuroblast cells sequentially express Hunchback (Hb), Seven-up (Svp), Kruppel (Kr), Pdm1/Pdm2 (Pdm) and Castor (Cas) (Kohwi et al., 2013). Expression
in the neuroblast is inherited and maintained in the daughter neuron after asymmetric division and functions to regulate neural subtype specific gene expression programs (Yu et al., 2006). The mechanism by which these transcriptional programs are initiated are still poorly understood, however perturbations in this temporal expression sequence affect both the fate the of postmitotic neuron subtype in which they are normally expressed as well as the subsequent generation of later born neural subtypes (Spindler and Hartenstein, 2010).

These examples of neural subtype specification in the vertebrate spinal cord and Drosophila larvae highlight the central role that transcriptional regulation plays in controlling cell fate and suggest this is a critical mechanism by which neural diversity is generated during nervous system development. The remainder of this thesis will focus on the functions of two closely related transcription factors Fezf1 and Fezf2 during development of both the CNS and PNS.

Fez family transcription factors

The forebrain embryonic zinc finger (FEZ) family is a highly conserved family of transcription factors that was first identified through their specific expression in the anterior neuroepithelium of Xenopus and zebrafish embryos (Shimizu and Hibi, 2009). In C. elegans and Drosophila there is a single Fez family homologue (Y38HA8.5 or Erm respectively) while the vertebrate Fez family contains two members, FEZF1 and FEZF2. Family members all share a N terminal Engrailed homology 1 (Eh) repressor domain and six C2H2 zinc-finger DNA binding domains
at the C terminal (Figure 1-1). Eh1 domains are known to interact with the Groucho/TLE family of transcriptional co-repressors (Hashimoto et al., 2000b). Combined with in vivo studies in model organisms, this suggests that these factors function as transcriptional repressors (Shimizu et al., 2010b).

Transcription of the Fez family is regulated by secreted factors during neuronal development. *Fezf1* was first identified though a screen for genes that are induced in response to overexpression of *Noggin* in *Xenopus* (Matsuo-Takasaki et al., 2000). Similarly, *Fezf2* was isolated by screening for genes that are up-regulated in zebrafish in response to overexpression of the Wnt inhibitor *Dkk1* (Hashimoto et al., 2000a). In mice expression of *Fezf1* and *Fezf2* is first detectable around embryonic day (E) 8.5 in the region corresponding to the future forebrain (Hirata et al., 2006a; Hirata et al., 2004). By mid-gestation, *Fezf1* and *Fezf2* show both overlapping and distinct expression in the forebrain and olfactory system. *Fezf1* is expressed in the main olfactory epithelum, amygdala, ventral thalamus and hypothalamus (Hirata et al., 2006a; Hirata et al., 2006b). In contrast, *Fezf2* is expressed in the vomeronasal organ, dorsal telencephalon, ventral thalamus and hypothalamus (Hirata et al., 2006b; Hirata et al., 2004). As development precedes expression of *Fezf1* becomes confined to the main olfactory epithelium, hypothalamus and amygdala while *Fezf2* is expressed in deep layers of the cerebral cortex, the vomeronasal organ, amygdala and hypothalamus (Hirata et al., 2006a; Molyneaux et al., 2005; Watanabe et al., 2009). The overlapping as well as distinct expression patterns of these genes underscore their broad functions during nervous system development.
**Evolution of the Fez family of transcription factors.** (A) Schematic of FEZF1 and FEZF2 proteins highlighting the strong evolutionary conservation of the engrailed homology (Eh) and zinc-finger DNA binding domains. Conservation is reported as percent homology to *Homo sapiens* FEZF1. (B) Phylogenetic tree of Fez family transcription factors. A single duplication event appears to have created the two FEZF proteins from a common ancestor. (C) Reference phylogenetic tree demonstrating the evolutionary relationship between species used in (A) and (B).
During olfactory system development, \textit{Fezf1} is expressed in olfactory sensory neurons (OSNs) as well as their precursors (Hirata et al., 2006b; Watanabe et al., 2009). Previous studies indicate that it is required for the maturation of OSNs as well as innervation of the olfactory bulb (Hirata et al., 2006b; Watanabe et al., 2009). OSNs that lack \textit{Fezf1} express decreased levels of the mature OSN marker OMP (Watanabe et al., 2009). Additionally, axons from these neurons fail to cross the cribriform plate and innervate the olfactory bulb (Hirata et al., 2006b; Watanabe et al., 2009). \textit{Fezf1} has also been implicated in the development of olfactory bulb interneurons. In \textit{Fezf1} mutant mice, organization of the olfactory bulb is significantly disrupted and specific interneuron subtypes are absent (Hirata et al., 2006b). However, since \textit{Fezf1} is not expressed in olfactory bulb interneurons, this suggests that these effects are non-cell autonomous.

The role of \textit{Fezf2} in development of the forebrain has been well documented. \textit{Fezf2} is expressed at high levels in ventricular zone progenitors and deep layer neurons of the cerebral cortex (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). In \textit{Fezf2} mutant mice, deep layer projection neurons fail to properly develop. Pyramidal neurons in layer five of the cortex fail to send projections to the brainstem and spinal cord (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). Instead, they adopt aspects of upper-layer intra-cortical projection neurons. These neurons express molecular markers for coritco-cortical projection neurons, extend axons across the corpus collusum and display electrophysiological profiles of these neurons (Chen et al., 2008). Several studies indicate that \textit{Fezf2} is not only
required for development of corticospinal motor neurons (CSMNs) but that \textit{Fezf2} expression levels are critical to proper development of the cerebral cortex. TBR1 is a transcription factor expressed in layer six corticothalamic projection neurons. In \textit{Trb1} mutant mice, corticothalamic projections are absent and instead layer six neurons extend ectopic projections toward the brainstem. This also includes an increase in \textit{Fezf2} expression in layer 6 suggesting a fate switch from corticothalamic neurons to subcerebral projection neurons (Han \textit{et al.}, 2011; McKenna \textit{et al.}, 2011). Additionally, several studies indicate that mis-expressing \textit{Fezf2} at artificially high levels in late cortical progenitors as well as immature neurons is sufficient to reprogram these cells to project subcerebral instead of colossal projection neurons (Chen \textit{et al.}, 2008; De la Rossa \textit{et al.}, 2013; Rouaux and Arlotta, 2012). Collectively this indicates that \textit{Fezf2} expression is both necessary and sufficient for the generation of subcerebral projection neurons.

\textit{Fezf1} and \textit{Fezf2} have also been implicated in controlling the neurogenic programs of neocortical progenitors during early development of the cerebral cortex. These factors were shown to bind the promoter of the pro-neural gene \textit{Hes5} and repress its transcription. In \textit{Fezf1}; \textit{Fezf2} mutant mice expression of \textit{Hes5} is increased in the forebrain while expression of the proneural gene \textit{Neurogenin2} is decreased (Shimizu \textit{et al.}, 2010b). Ultimately, this results in the failed differentiation basal progenitor cells and early born deep-layer neurons.

The above examples indicate that Fez family of transcription factors play critical roles in the development and fate specification of multiple neuronal subtypes.
during nervous system development. In the following pages I will describe my work to understand how neuronal cell fates are specified in the both the olfactory system and cerebral cortex, focusing on the roles of Fezf1 and Fezf2. These studies address the following questions: 1) How are closely related but distinct neuronal subtypes specified during development? 2) How can seemingly homogeneous neural progenitors generate neurons with different molecular, morphological and functional identities? 3) What role do genome organization and non-coding DNA elements play in neuronal subtype specification?
Chapter 2: *Fezf1* and *Fezf2* are required for olfactory development and sensory neuron identity

**Introduction**

To perceive their chemical environment, mice coordinate the functions of the main and accessory olfactory systems. The main olfactory system is composed of the main olfactory epithelium (MOE) and the main olfactory bulb, which are involved in odorant detection. Alternatively, the accessory olfactory system consists of the vomeronasal organ (VNO), the Grüneberg ganglion, the septal organ of Masera and the accessory olfactory bulb (Munger *et al.*, 2009). The best studied of these accessory sense organs, the VNO, is primarily involved in pheromone detection (Dulac and Torello, 2003). Thus, the MOE and VNO are considered functionally and anatomically distinct organs.

During mid-gestation the MOE and VNO both originate from the bilaterally symmetrical olfactory pits, which are invaginations of epithelial and underlying mesenchymal cells (Figure 2-1A) (Balmer and LaMantia, 2005). At E9.5 the olfactory pits first develop as a thickening of the frontal epithelial cell layer of the developing embryo known as the olfactory placode. The olfactory placode begins to show signs of neural differentiation such as β-tubulin and NCAM expression, and by E10.5 has undergone an initial invagination to produce the two olfactory pits (LaMantia *et al.*, 2000) (Figure 2-1A). By E12.5 the olfactory pits undergo a second invagination in the ventromedial wall that produces the VNO (Figure 2-1B). The
**Figure 2-1**

**A diagram depicting the developmental origins of the main olfactory epithelium (MOE) and vomeronasal organ (VNO) from the olfactory pit.** All schematics shown depict a coronal orientation. (A) At E10.5 the olfactory pit (shown in yellow) is morphologically discernible but does not show any distinction between the regions that will give rise to the MOE and VNO. (B) By E12.5 the pit has undergone an initial invagination, distinguishing the dorsal region that will give rise to the MOE (purple) from the ventromedial region that will become the VNO (green). (C) By E14.5 the VNO has separated from the pit and become anatomically distinct. (D) This spatial and molecular distinction is clear at birth (P0), with both the MOE and VNO projecting axons to innervate the olfactory bulb (OB) (grey). Diagrams are not drawn to scale.
developing VNO separates from the olfactory pit and becomes a spatially distinct organ from the MOE by E14.5 (Figure 2-1C-D) (Balmer and LaMantia, 2005).

The mature MOE and VNO consist of a sensory neuron layer and an apical layer of supporting cells (Kawauchi et al., 2004). The MOE also contains a basal layer of progenitor cells (Beites et al., 2005). Tremendous progress has been made toward determining the signaling events in sensory neurons of the MOE and VNO (Mombaerts, 2004). The mouse genome encodes about 1,000 G protein-coupled olfactory receptor genes (ORs) and each sensory neuron of the MOE (OSN) expresses only one of these ORs. Binding of odorant molecules to an OR activates the OSN-specific G protein G\textsubscript{olf}, which then activates adenylate cyclase III (Jones and Reed, 1989). The resulting increase in cAMP levels leads to the opening of olfactory cyclic nucleotide-gated channels and the propagation of an action potential (Liman and Buck, 1994). In addition to the Ors, the mouse genome encodes two classes of G protein-coupled pheromone receptors: V1Rs and V2Rs. Sensory neurons in the apical zone of the VNO (VSNs) express V1Rs and the G protein subunit G\textsubscript{a2i}, while VSNs in the basal zone express V2Rs and G\textsubscript{o}. Binding of pheromone molecules to V1Rs or V2Rs likely leads to the activation of the VNO-specific cation channel Trpc2.

Axons from OSNs innervate the main olfactory bulb (MOB) while axons from the VSNs project to the accessory olfactory bulb (AOB) (Mombaerts, 2004).

Significant advances have been achieved in understanding transcriptional control of OSN development. Mash\textsubscript{1} is an important proneural gene in olfactory neurogenesis. It is expressed in the progenitor cells of MOE but not in differentiated
OSNs (Cau et al., 1997; Gordon et al., 1995). In Mash1−/− mice, the number of mitotic progenitors and neurons are reduced in the MOE, and expression of the sustentacular cell marker Kitl is dramatically increased (Cau et al., 2002; Cau et al., 1997; Guillemot et al., 1993; Murray et al., 2003), indicating that Mash1 is critical for neuronal determination in the MOE. Two transcription factors, Hes1 and Wt1 (KTS) have been shown to affect the expression of Mash1 (Cau et al., 2000; Wagner et al., 2005), and mice carrying mutations in these genes show abnormal MOE neurogenesis (Cau et al., 2002; Cau et al., 2000; Wagner et al., 2005). Many transcription factors, including Ngn1 (Cau et al., 2002; Cau et al., 1997) and Lhx2 (Cau et al., 2002; Hirota and Mombaerts, 2004; Kolterud et al., 2004) act downstream of Mash1 and are important for OSN differentiation. Additional transcription factors, such as members of O/E family (Wang et al., 2002; Wang et al., 2004; Wang et al., 1997), MeCP2 (Matarazzo et al., 2004; Ronnett et al., 2003), Dlx5 (Levi et al., 2003; Long et al., 2003), and KLF7 (Laub et al., 2001; Luo et al., 1995; Tanaka et al., 2002), function further downstream in OE development and regulate OSN maturation and axonal projections to the OB. Although these studies have focused on MOE and OSN development, many of these transcription factors are also expressed in the VNO, suggesting that similar mechanisms may regulate VSN development. Despite this progress, the mechanisms regulating the early cell fate decisions that generate the MOE and VNO, two distinct organs that develop from a common primordium, are not well understood.
Fez family zinc-finger proteins 1 and 2 (FEZF1 and FEZF2) are two closely related transcription factors expressed early during mouse development that are important for brain development and cell identity. *Fezf2* is required for proper fate specification of layer 5 subcortical projection neurons in the cerebral cortex (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Molyneaux et al., 2005), while *Fezf1* is essential for proper development of the OB and MOE (Hirata et al., 2006b; Watanabe et al., 2009). Furthermore, both *Fezf1* and *Fezf2* are required for regulation of forebrain size and patterning during early development (Hirata et al., 2006a; Shimizu et al., 2010a). Here we report the functions of *Fezf1* and *Fezf2* in establishing MOE neuronal identity and VNO development, respectively. We found that *Fezf1* and *Fezf2* show distinct expression patterns in the developing olfactory system. *Fezf1* is expressed strongly in the MOE and weakly in the VNO, while *Fezf2* is specifically and highly expressed in the VNO. Analysis of *Fezf1*−/− and *Fezf2*−/− mice indicates these genes are necessary for proper cell identity of olfactory sensory neurons (OSNs) and maintenance of the VNO, respectively. In *Fezf1* deficient mice, OSNs fail to mature and express VNO-enriched neuronal markers. In contrast, *Fezf2* mutant animals lack a VNO at birth. These results identify *Fezf1* and *Fezf2* as important regulators of olfactory system development and sensory neuron identity.
Results

Fezf1 and Fezf2 show distinct expression patterns during olfactory development

To investigate the expression patterns of Fezf1 and Fezf2 in the olfactory system we performed in situ hybridization using cRNA probes. Fezf1 expression was first detected in the olfactory placode at E9.5 (Figure 2-2A). At E10.5, Fezf1 mRNA was detected in the invaginating olfactory pit and expression continued postnatally in the olfactory system, with higher expression in the MOE and lower expression in the VNO (Figure 2-2B, D, and data not shown). Fezf2 mRNA was first detected in the olfactory pit at E10.5 and was localized to the ventromedial region from which the future VNO is derived (Figure 2-2C). After the VNO separated from the olfactory pit, Fezf2 mRNA was detected exclusively in the VNO and this expression pattern was maintained until postnatal stages (Figure 2-2E, and data not shown).

The in situ hybridization data demonstrated that Fezf1 and Fezf2 preferentially demarcate two distinct parts of the olfactory system: MOE and VNO. To confirm the in situ hybridization results and further investigate the expression of Fezf1 and Fezf2 at the cellular level, we used two different reporter mouse lines. A Fezf1+/− strain generated in our laboratory that contains both EGFP and PLAP coding sequences under the control of the endogenous Fezf1 promoter (Figure 2-3) was used to investigate Fezf1 expression. In the Fezf2 mutant allele generated previously (Chen et al., 2005a; Chen et al., 2008), even though an EGFP-ires-PLAP cassette was used to replace the genomic region encoding the Fezf2 open reading frame, EGFP protein
Expression of *Fezf1* and *Fezf2* transcripts during olfactory system development. Radioactive *in situ* hybridization was used to detect *Fezf1* (A, B, and D) or *Fezf2* (C, and E) expression. At E9.5 *Fezf1* was detected in the olfactory placode (arrowheads in A). At E10.5 it is detected throughout the newly formed olfactory pits (arrowheads in B). *Fezf1* remains strongly expressed in the MOE (arrowheads in D) and weakly in the VNO (arrows in D) at E14.5 and later stages (D, and data not shown). Alternatively, *Fezf2* is specifically expressed in the VNO throughout olfactory system development (arrows in C and E). A, sagittal section; B-E, coronal sections. The scale bar in A is 25 μm and in E is 100 μm.
**Fezf1 knockout strategy.** The endogenous Fezf1 locus was replaced with a cassette containing *EGFP*-IRES-*hPLAP* (A) and transmission of the targeted allele was confirmed by PCR using primers P1 and P2 (for wild type allele), P3 and P4 (for mutant allele) (B). Appropriate expression of the targeted Fezf1 allele was confirmed by radioactive *in situ* hybridization to detect PLAP expression in *Fezf1*+/− (D), but not in wild type mice (C). The scale bar in D is 100 μm.
was not detected in the mice carrying the mutant allele. Thus, to examine \textit{Fezf2} expression at a cellular level, instead of using the PLAP marker in the \textit{Fezf2} mutant allele, we utilized a \textit{Fezf2-EGFP} transgenic mouse line carrying an EGFP marker under the control of the \textit{Fezf2} promoter, which was produced by the GENSAT project using a modified bacterial artificial chromosome (Gong \textit{et al.}, 2003). Consistent with the \textit{in situ} hybridization results, at E10.5 and E12.5, \textit{Fezf1-EGFP} was strongly expressed in cells across the full extent of the olfactory epithelium including the region that gives rise to the VNO (Figure 2-4A, B, and A’ for MOE, G, H and G’ for VNO). As development proceeded, \textit{Fezf1-EGFP} expression remained high in the MOE (Figure 2-4C-F) and decreased in the VNO (Figure 2-4I-L). After E14.5, \textit{Fezf1-EGFP} expression within the MOE became restricted to OSNs and the basal progenitor cell layer in the MOE and was excluded from the supporting cell layer (Figure 2-4D-F, and F’). \textit{Fezf1-EGFP} expression in VNO decreased (Figure 2-4J-L), and was detected in very few neurons in the VNO at P0 (Figure 2-4L and L’).

In contrast to \textit{Fezf1-EGFP}, expression of \textit{Fezf2-EGFP} was restricted to the VNO. At E10.5, when VNO morphogenesis is first discernible as a local thickening of the neural epithelium in the ventromedial region of the olfactory pit, high \textit{Fezf2-EGFP} expression was detected in the region that will become the VNO (Figure 2-4S, and S’). To our knowledge this identifies \textit{Fezf2} as the earliest marker distinguishing the region of the olfactory pit that gives rise to the VNO from the rest of the olfactory pit. \textit{Fezf2-EGFP} expression persisted in the VNO during its separation from the MOE (Figure 2-4T and U). Prior to E14.5 \textit{Fezf2-EGFP} was expressed at high levels in most
Expression patterns of *Fez1* and *Fez2* during development of the olfactory system detected by EGFP reporter expression. GFP immunohistochemistry was used to visualize cells expressing either *Fez1*-EGFP (A-L, A’, F’, G’, L’) or *Fez2*-EGFP (M-X, M’, R’, S’, X’). Nuclei were visualized using SYTOX orange. At E10.5 and E12.5, *Fez1*-EGFP is expressed throughout the future MOE and VNO (A, B, G, H, A’, and G’). After E14.5 *Fez1*-EGFP expression starts to decrease in the VNO and becomes restricted to progenitor and neuronal cell layers of the MOE (D-F, J-L, F’, and L’). This transition is complete by E18.5 and persists postnatally (E, F, K, L, F’, and L’). In contrast to the *Fez1*-EGFP expression pattern, *Fez2*-EGFP expression is restricted to the VNO throughout olfactory system development (M-X, M’, S’, R’, and X’). At E10.5, when the VNO is first starting to differentiate from the olfactory pit, *Fez2*-EGFP is strongly expressed throughout cells in the ventromedial anlage that will give rise to the VNO (S, S’). Beginning at E14.5, *Fez2*-EGFP expression undergoes a dynamic transition and becomes restricted to the sustentacular cell layer of the VNO (O-R; U-X, R’, X’). This expression pattern of *Fez2*-EGFP is maintained postnatally (R, X, R’, X’). For all images, EGFP expression is shown in green and SYTOX orange staining is in purple. Images in A’, F’, G’, L’, M’, R’, S’ and X’ represent the enlarged regions shown in the white boxes in panels A, F, G, L, M, R, S and X. The scale bar in X is 75 µm and in X’ is 30 µm.
cells of the developing VNO (Figure 2-4S, T, S'). However, after E14.5 Fezf2-EGFP expression became restricted to the apical sustentacular cell layer of the VNO (Figure 2-4V-X, X’). This dynamic transition was complete by E18.5 and persisted until postnatal stages (Figure 2-4X, X’ and data not shown). No Fezf2-EGFP expression was detected in cells of the MOE at any age (Figure 2-4M-R, M’, and R’). Taken together, the in situ hybridization data and cell labeling with Fezf1-EGFP and Fezf2-EGFP markers demonstrate that expression of Fezf1 and Fezf2 is largely mutually exclusive; Fezf1 is preferentially expressed in the MOE and Fezf2 is exclusively expressed in the VNO during their development. Moreover, after E14.5 Fezf1 and Fezf2 become restricted to progenitors and neurons of the MOE and sustentacular cells of the VNO, respectively.

The mechanisms regulating segregation of the MOE and VNO from a common olfactory primordium remains unknown. Because Fezf2 plays an essential role in specifying neuronal identity in the developing cerebral cortex, preferential expression of Fezf1 in the MOE and exclusive expression of Fezf2 in the VNO suggested that these two genes are involved in specifying MOE and VNO cell identities. We tested this possibility by analyzing development of the olfactory system in Fezf1−/−, Fezf2−/−, and Fezf1−/−; Fezf2−/− mice.

**Incomplete maturation of the MOE in Fezf1−/− and Fezf1−/−; Fezf2−/− mice**

If Fezf1 is required for specification of MOE identity, Fezf1−/− mice should exhibit abnormal olfactory system development. Indeed, defects in the MOE of Fezf1−/−
mice have been described previously (Hirata et al., 2006b; Watanabe et al., 2009). Consistent with published data, when we examined the olfactory axon projections in Fezf1\(^{-/-}\) mice using the PLAP marker which was knocked into the Fezf1 mutant allele, we found that OSNs were present in Fezf1\(^{-/-}\) mice but their axonal projections failed to reach the olfactory bulb and instead formed a fasciculated bundle (compare panels 2-5A, B). This defect was not observed in Fezf2\(^{-/-}\) animals (Figure 2-5C), and Fezf1\(^{-/-}\); Fezf2\(^{-/-}\) mice showed no exacerbation of the OSN axonal defects in the Fezf1\(^{-/-}\) animals (Figure 2-5B, D).

To characterize further the defects in MOE development in Fezf1\(^{-/-}\) mice, we performed immunohistochemistry to investigate expression of two markers for mature OSNs: Olfactory Marker Protein (OMP) and the MOE expressed G-protein alpha subunit G\(\alpha_{\text{olf}}\). Consistent with a previous report (Watanabe et al., 2009), OMP expression was dramatically decreased in OSNs of Fezf1\(^{-/-}\) mice at postnatal day 0 (P0) (Figure 2-6A and B) and this same phenotype was observed in Fezf1\(^{-/-}\); Fezf2\(^{-/-}\) mice (Figure 2-6D). Similar to the defects in OMP expression, we also observed a significant decrease in the expression of G\(\alpha_{\text{olf}}\) in OSN dendrites from both Fezf1\(^{-/-}\) and Fezf1\(^{-/-}\); Fezf2\(^{-/-}\) animals (Figure 2-6E, F and H). Immunostaining for neural cell adhesion molecule (NCAM) and L1 cell adhesion molecule (L1) did not show any gross defects in MOE organization (Figure 2-6A-H), suggesting that a pan-neuronal identity was developed in the MOE of Fezf1 mutant mice. Further, the defects in OMP and G\(\alpha_{\text{olf}}\) expression were not observed in the MOE of Fezf2\(^{-/-}\) mice (Figure 2-6C,G), suggesting that Fezf1, and not Fezf2, is required for MOE-specific maturation.
**Fezf1 is required for OSN axons to reach the olfactory bulb.** (A-D) PLAP staining was used to visualize Fezf1 and Fezf2 expressing cells and their projections. In Fezf1<sup>+/−</sup>; Fezf2<sup>−/−</sup> (A) and Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> (C) mice the axons of OSNs (arrows) extended from the MOE and innervated the OB (asterisk). However in Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> (B) and Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> (D) mice OSN axons (arrowheads) were unable to penetrate the cribriform plate and innervate the OB. Note also the OB is not present in the Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> mice. The very dark PLAP staining in the OB of Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> mice is due to PLAP-labeled axons from deep-layer neurons of the cerebral cortex which projected ectopically into the olfactory bulb. The scale bar in D is 200 μm.
The OSNs in the MOE of *Fezfl* mice fail to mature. Immunohistochemistry shows a reduction in olfactory marker protein (OMP) expression in the MOE of *Fezfl* (B) and *Fezfl*; *Fezfl* (D) compared with control (A) and *Fezfl* mice (C). Similarly, Gas/olf expression is markedly reduced in the dendrites (white arrowheads in E-H) of *Fezfl* (F) and *Fezfl*; *Fezfl* (H) when compared with control (E) and *Fezfl* mice (G). Neural cell adhesion molecule (NCAM) and L1 cell adhesion molecule (L1) expression both appeared unaffected in *Fezfl* (B and F), *Fezfl* (C and G) and *Fezfl*; *Fezfl* (D and H) animals. Reduction in CNGA2 expression was visualized by radioactive *in situ* hybridization for the control and *Fezfl* mutant MOE (I and J). Non-radioactive *in situ* hybridization using probes corresponding to a mixture of olfactory receptors (ORs) (K and L) or trace amine associated receptors (TAARs) (M and N) showed loss of expression in *Fezfl* animals. Scale bars are H: 75 μm; J: 200 μm; and N: 100 μm.
We next performed in situ hybridization to examine expression of MOE signal transduction components. These included the cyclic nucleotide gated ion channel CNGA2 (Figure 2-6I, J), a subset of odorant receptors (ORs) (Figure 2-6K, L) and a subset of trace amine-associated receptors (TAARs) (Figure 2-6M, N). Consistent with our observations for OMP and Goαs/olf, levels of these transcripts were decreased in the Fezf1+/− MOE compared with controls (Figure 2-6I-N). Taken together, these data demonstrate that proper MOE-specific development and maturation requires Fezf1 and is not affected by loss of Fezf2.

**VNO degeneration in Fezf2−/− and Fezf1+/−; Fezf2−/− mice**

The early expression of both Fezf1 and Fezf2 in the VNO suggested they function during VNO development. To test this, we examined VNO development in Fezf1+/−, Fezf2−/−, and Fezf1−/−; Fezf2−/− mice. Taking advantage of the IRES-PLAP knocked into the Fezf1 and Fezf2 mutant alleles, we used alkaline phosphatase staining to visualize Fezf1 and Fezf2 expressing cells and their axonal projections. At E13.5 the ventromedial region of the olfactory pit separates to give rise to the VNO. We observed normal segregation of the VNO in Fezf1+/−, Fezf2−/−, and Fezf1−/−; Fezf2−/− mice (Figure 2-7A-D) but found that VNOs in Fezf1+/−; Fezf2−/−, and Fezf1−/−; Fezf2−/− mice (Figure 2-7C, D) appeared smaller than in Fezf1+/−; Fezf2+/− or Fezf1−/−; Fezf2+/− mice (Figure 2-7A, B). Strikingly, PLAP-positive cells were totally absent from Fezf1+/−; Fezf2−/− and Fezf1+/−; Fezf2−/− VNOs at P0 and only the surrounding cartilage and supporting tissues remained (Figure 2-7G, H). Immunostaining for NCAM
**Figure 2-7**

VNO degeneration in *Fezf2*−/− mice. (A-H) PLA2 staining was used to visualize Fez1 and Fezf2 expressing cells of the olfactory system. At E13.5 the VNO properly separated from the olfactory pit in Fez1+/+; Fezf2−/−, Fez1−/−; Fezf2−/−, and Fez1−/−; Fezf2−/− mice (A-D). However, in Fezf2−/− and Fez1−/−; Fezf2−/− animals the VNO is smaller (compare C, D with A, B). This defect is exacerbated by P0, with a complete absence of PLA2-positive cells in Fez1−/−; Fezf2−/−, and Fez1−/−; Fezf2−/− animals (compare G, H to E, F). The loss of neurons in the Fezf2 mutant VNO was confirmed by NCAM staining at P0 (I and J). NCAM+ cells (*) and their axons (arrowhead) are clearly visible in the Fezf2−/− but not in the Fezf2−/− VNO. Quantification of phospho-histone H3 (PHH3+) and cleaved-caspase 3+ cells indicates a significant decrease in proliferation (average of 3.38 vs. 2.31 per VNO section, p = 0.0269) (K) and increase in apoptosis (average of 1.28 vs. 2.48 per VNO section, p = 0.0003) (L). For Fezf2−/−, n = 80 sections and for Fezf2−/−, n = 52 sections. The scale bar in H is 100 μm and in J is 75 μm.
confirmed this loss of VNO neurons in Fezf1<sup>+/−</sup>; Fezf2<sup>−/−</sup> and Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> mice, as NCAM-expressing cells were clearly visible in the VNO of Fezf2<sup>−/−</sup> but not Fezf2<sup>−/−</sup> mice (Figure 2-7I, J). Although Fezf1 was detected at low levels in the developing VNO (Figure 2-4G-L and G’, L’), we did not observe a significant reduction of PLAP staining in the VNO of Fezf1<sup>−/−</sup> mice compared to Fezf1<sup>+/−</sup> (Figure 2-7A, B, E, and F). Furthermore, degeneration of the nascent VNO in Fezf2<sup>−/−</sup> mice was not exacerbated by deletion of Fezf1 (compare Figure 2-7D, H to 7C, G). Taken together, these results indicate that the VNO is initially patterned but subsequently degenerates in Fezf2 deficient mice. Thus, Fezf2 but not Fezf1 is required for maintenance of VNO cells.

**Altered proliferation and apoptosis in the VNO of Fezf2<sup>−/−</sup> mice**

VNO degeneration in Fezf2<sup>−/−</sup> mice could be the result of decreased proliferation, increased cell death or both factors. To investigate these possibilities we performed immunohistochemistry to assay the number of phosphorylated histone H3 (PHH3)-positive cells and cleaved caspase 3-positive cells. This allowed us to determine the number of mitotic and apoptotic cells, respectively. Upon comparison of serial sections from E12.5 VNOs from Fezf2<sup>+/−</sup> and Fezf2<sup>−/−</sup> mice we observed a statistically significant decrease in proliferation and an increase in cell death. Compared to Fezf2<sup>+/−</sup> VNOs, Fezf2-deficient VNOs showed a 1.5 fold average decrease in PHH3<sup>+</sup> cells (Figure 2-7K) and 1.9 fold average increase in cleaved-caspase 3<sup>+</sup> cells per section (Figure 2-7L). These data indicate that the VNO cells fail to proliferate in Fezf2<sup>−/−</sup> mice and instead undergo extensive cell death.
Transcriptome analysis of Fezf1-/- OSNs

To understand more extensively the function of Fezf1 in olfactory system development, we compared gene expression profiles for Fezf1+/+ and Fezf1-/- olfactory cells. We purified EGFP-expressing cells from the MOE of E18.5 Fezf1+/+ and Fezf1-/- animals, using the EGFP marker present in the Fezf1 mutant allele. Fluorescence activated cell sorting (FACS) allowed us to obtain a population of Fezf1-EGFP expressing cells that was greater than 90% pure. Total RNA from these cells was subjected to expression profiling using Affymetrix microarrays. A pairwise comparison of expression profiles between Fezf1+/+ and Fezf1-/- cells yielded a list of 152 up-regulated and 545 down-regulated transcripts. From this list we identified genes as either MOE- or VNO-enriched based upon literature searches and the online expression databases Alan Brain Atlas (www.brain-map.org) and GenePaint (www.genepaint.org). Among the down-regulated transcripts, 74% corresponded to MOE-enriched genes (Figure 2-8A), supporting our observation that the MOE fails to mature properly in Fezf1-/- mice. Strikingly, 19% of transcripts up-regulated in Fezf1-/- cells corresponded to VNO-enriched transcripts (Figure 2-8A). These genes encode VNO receptors, ion channels, components of VNO signal transduction and VNO-specific transcription factors (Table S1, available online). Thus, gene expression analysis demonstrates that in Fezf1-/- mice the MOE fails to mature properly and instead adopts a VNO-like transcriptional identity. Unfortunately, due to the early degeneration of the VNO in Fezf2-/- mice we were not able to perform a similar analysis for Fezf2-expressing VNO cells.
**Fezf1** MOE acquires a VNO-like transcriptional identity. Microarray analysis of E18.5 **Fezf1** cells showed that 74% of down-regulated transcripts and 19% of up-regulated transcripts correspond to MOE- and VNO-enriched transcripts, respectively (A). *In situ* hybridization confirming mis-expression of VNO-enriched transcripts in the MOE of **Fezf1** mice (B-G). Transient receptor potential cation channel 2 (**Trpc2**) (B, C), some vomeronasal receptor class 1 genes (**V1Rs**) (D, E), and some vomeronasal receptor class 2 genes (**V2Rs**) (F, G) were mis-expressed in the MOE of **Fezf1** mutant animals. **Trpc2** expression was visualized by radioactive *in situ* hybridization. Expression of **V1Rs** and **V2Rs** was visualized by digoxigenin *in situ* hybridization. Scale bars in C and G are 100 μm.
The \textit{Fezf1}^{-/-} MOE displays a mixed cell identity

Our microarray experiment suggested that cells in the MOE of \textit{Fezf1}^{+/+} mice adopted the transcriptional identity of the VNO. To examine this further we performed \textit{in situ} hybridization with VNO-enriched markers on olfactory tissues from \textit{Fezf1}^{+/+} and \textit{Fezf1}^{-/-} animals. Consistent with the microarray data, we observed that the MOE in \textit{Fezf1}^{-/-} mice ectopically expressed components of the VNO signal transduction pathway. The transient receptor potential cation channel 2 gene (\textit{Trpc2}) is expressed by all vomeronasal sensory neurons (VSNs) and is required for their function (Stowers \textit{et al.}, 2002). In \textit{Fezf1}^{+/+} mice, \textit{Trpc2} is strongly expressed in the VNO and weakly in the MOE (Figure 2-8B). Strikingly, we observed significant up-regulation of \textit{Trpc2} in the MOE of \textit{Fezf1}^{-/-} animals (Figure 2-8C). Vomeronasal receptor class 1 and 2 genes (V1Rs and V2Rs, respectively) are important for pheromone detection and their expression is strongly enriched in the VNO. When we compared the expression of V1Rs and V2Rs in \textit{Fezf1}^{+/+} (Figure 2-8D, F) and \textit{Fezf1}^{-/-} mice (Figure 2-8E, G) we observed their ectopic expression in the MOE. The \textit{in situ} hybridization data show that the MOE of \textit{Fezf1}^{-/-} animals expresses VNO-enriched genes. However, since many MOE-enriched genes are still expressed in \textit{Fezf1}^{-/-} mice (Table S1), these results indicate that in \textit{Fezf1}^{-/-} mice the MOE only partially acquires a VNO-like identity.

\textit{Fezf1} and \textit{Fezf2} are not mutually repressive

Two possible mechanisms might have contributed to the expression of VSN-
specific genes in the MOE of Fezf1^{-/-} mice. The first possibility was that Fezf1 might regulate the proper sorting and segregation of VNO progenitor cells from MOE progenitor cells. In the absence of Fezf1 function, some VNO progenitors might remain in MOE, leading to VSN-specific gene expression in the MOE. The second possible mechanism was that Fezf1 and Fezf2 might promote MOE and VNO identities, respectively, and accomplish this through mutual repression of each other, such that FEZF1 prevented expression of Fezf2 in the MOE and FEZF2 inhibited expression of Fezf1 in the VNO. Since Fezf2 is a VNO-specific progenitor cell marker, if either possibility was true, we expected Fezf2 expression to be increased in the MOE of Fezf1^{-/-} mice. Thus we performed in situ hybridization at early and late developmental stages on Fezf1^{-/-} and Fezf2^{-/-} mice. We first examined expression of Fezf1 in Fezf2^{+/+} and Fezf2^{-/-} mice at E13.5. At this age we found no evidence demonstrating that loss of Fezf2 affects expression of Fezf1 (Figure 2-9A, B). This observation is consistent with the fact Fezf2 is not expressed in the MOE during development. Unfortunately, the early degeneration of the VNO in Fezf2^{-/-} mice precluded the examination of Fezf1 expression in the Fezf2 mutant VNO at P0.

Next we assayed Fezf2 expression in Fezf1^{+/+} and Fezf1^{-/-} mice at E13.5 and P0. Similar to the results for Fezf1 expression, we did not observe any significant change in the expression levels of Fezf2 in the MOE or VNO after loss of Fezf1 (Figure 2-9C-F). Consistent with these results, our microarray analysis did not show any increase in Fezf2 expression in purified Fezf1^{-/-} MOE cells at E18.5 (Table S1, available online).
Fezf1 and Fezf2 are not mutually repressive. Radioactive in situ hybridization is used to visualize Fezf1 (A, B) or Fezf2 (C-F) transcripts. At E13.5 expression of Fezf1 is not increased in Fezf2−/− mice (A, B). Similarly, loss of Fezf1 has no effect on expression of Fezf2 at E13.5 or P0 (C-F). The VNO is marked by arrowheads (A, B). The MOE is indicated by asterisks (C-F). Scale bars in D and F are 100 µm.
Analysis of Fezf1<sup>+/−</sup> and Fezf2<sup>+/−</sup> mice suggests that VNO progenitor cells segregate properly from MOE progenitors in the Fezf1<sup>+/−</sup> mice, and Fezf1 does not promote MOE neuronal fate by repressing Fezf2 expression in the MOE. Furthermore, olfactory development in the Fezf1<sup>−/−</sup>; Fezf2<sup>−/−</sup> mice was the sum of defects observed in Fezf1<sup>−/−</sup> and Fezf2<sup>−/−</sup> single mutant mice: expression of VNO-enriched genes is up-regulated in the MOE and the VNO quickly degenerates around E12.5-E13.5. Thus, both transcriptional and genetic analysis demonstrated that Fezf1 and Fezf2 do not exhibit mutually repressive interactions during olfactory development.

**Fezf1 and Fezf2 can function interchangeably in cell fate specification during development of the cerebral cortex**

The lack of genetic and molecular evidence for cross-inhibition between Fezf1 and Fezf2 prompted us to explore other possible mechanisms underlying their functions. The sequences of FEZF1 and FEZF2 proteins show a high degree of similarity. They both contain a putative C-terminal DNA binding domain consisting of 6 C<sub>2</sub>H<sub>2</sub> zinc-finger motifs and a N-terminal region with homology to the engrailed repressor domain, which can interact with the groucho/TLE family of co-repressors (Figure 10A) (Shimizu and Hibi, 2009). In fact, the zinc-finger motifs of these two proteins are 97% identical (Figure 2-10A). This high degree of similarity in their DNA binding domains suggests that FEZF1 and FEZF2 have the capacity to bind the same DNA regulatory sequences and likely regulate similar sets of genes.
**Fez1** and **Fez2** can function interchangeably during cell fate specification in vivo. (A) A schematic presentation of identifiable FEZF1 and FEZF2 protein domains showing their N-terminal engrailed homology domain (Eh) and C-terminal C2H2 zinc-finger motifs. An alignment of the zinc-finger motifs shows only three amino acid differences (*"*), corresponding to 97% identity between the two proteins. (B-L) Misexpression of **Fez1** in corticocortical projection neurons of the cerebral cortex is sufficient to redirect their axons subcortically, similar to previously reported results for **Fez2** misexpression (Chen et al., 2008). When **Fez1** and **EGFP** were electroporated into layer 2/3 neurons in utero (H–L) their axons are redirected to the striatum (J), thalamus (K) and pons (L). No GFP+ axons were detected in these brain regions when only **EGFP** was misexpressed (B–F). Our model predicts that **Fez1** promotes MOE development and **Fez2** promotes VNO development by preventing progenitors and sensory neurons of the MOE and progenitors and supporting cells of the VNO from assuming a VNO sensory neuron identity (M). The diagram in A is not drawn to scale. The scale bar in H is 1 mm and in L is 75 μm.
The expression patterns of Fezf1 and Fezf2 during olfactory system development and the phenotype of Fezf1−/− mice are consistent with the hypothesis that they regulate common gene targets. As development of the olfactory system proceeds, Fezf1 and Fezf2 are down-regulated in VSNs while their expression in OSNs and VNO supporting cells increases. Additionally, the up-regulation of VSN-enriched genes in the MOE of Fezf1−/− mice suggests that both Fezf1 and Fezf2 might function to repress VSN-enriched gene expression in the MOE and supporting cells of the VNO. Thus, we hypothesized that Fezf1 and Fezf2 may function to inhibit a VSN fate within MOE neurons and VNO supporting cells, respectively (Figure 2-10M). To test this, we performed a mis-expression assay to ascertain whether Fezf1 and Fezf2 can function interchangeably in cell fate specification. Instead of using the olfactory system, we performed this assay in the developing cerebral cortex, since this brain region is much more amenable for surgical manipulation to test gene function in vivo.

During development of the cerebral cortex, Fezf1 is not expressed in cortical neurons (Hirata et al., 2006a; Shimizu et al., 2010a). However, Fezf2 is expressed in subcortical projection neurons of the cerebral cortex and is both necessary and sufficient for the development of subcortical axons (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Molyneaux et al., 2005). Previously we have demonstrated that misexpression of Fezf2 in corticocortical projection neurons prevents these neurons from sending axons to other cortical areas (their normal axon targets), and instead redirects their axons to subcortical targets such as the thalamus and spinal cord (Chen et al., 2008). To test whether FEZF1 and FEZF2 can regulate similar sets
of genes, we ectopically expressed *Fezf1* in corticocortical projection neurons and assayed the effects on their axonal projections. Plasmids encoding *Fezf1* under control of the cytomegalovirus (CMV) early gene promoter and chicken β-actin enhancer were introduced into corticocortical projection neurons at E15.5 using *in utero* electroporation. Additionally, plasmids encoding the EGFP protein under the same promoter and enhancer were co-electroporated to label cell bodies and axons of the electroporated neurons. Following surgery the embryos were returned to the abdomens of pregnant female mice and allowed to develop to full term. Brains from the electroporated embryos were collected at P5. In brains electroporated with EGFP plasmids alone, GFP labeled axons were present in the striatum (Figure 2-10D) but not in the thalamus (Figure 2-10E) or the pons (Figure 2-10F), which are the axonal targets of subcortical neurons. Strikingly, in brains electroporated with *Fezf1* and EGFP expressing plasmids, many GFP labeled axons are present in the striatum (Figure 2-10J), thalamus (Figure 2-10K), and pons (Figure 2-10L), similar to the effect of mis-expressing *Fezf2* in corticocortical projection neurons (Chen *et al.*, 2008). This result demonstrates that *Fezf1* and *Fezf2* can regulate expression of common genes *in vivo*.

**Discussion**

The mechanisms specifying the MOE and VNO, two functionally and anatomically distinct organs, from a common olfactory pit have not been well defined. In this study we focused on the functions of two zinc-finger transcription
factors Fezf1 and Fezf2 in murine olfactory development. We found that Fezf2 is required for survival and proliferation of VNO progenitors and that Fezf1 regulates the maturation and identity of MOE sensory neurons.

During development, Fezf1 and Fezf2 are expressed in the olfactory pit at an early stage and demarcate distinct regions of the olfactory primordium. While Fezf1-expressing cells are distributed along the full length of the olfactory pit, Fezf2 expression is restricted to the ventromedial region that gives rise to the VNO, making Fezf2 the earliest marker for future VNO cells. Immediately after the VNO segregates from the MOE at E13.5, Fezf1 is expressed in both the MOE and VNO. After E14.5, while Fezf1 expression in OSNs and progenitors of the MOE steadily increases, its expression in the VNO decreases, and very few VNO sensory neurons still express Fezf1 at birth. Conversely, Fezf2 expression in the VNO is dynamic; prior to E14.5, Fezf2 is expressed throughout the VNO neuroepithelium. After E14.5, when supporting cells and VSNs segregate into distinct layers, Fezf2 expression becomes restricted to the sustentacular cell layer and is excluded from differentiating VSNs. These specific and dynamic expression patterns of Fezf1 and Fezf2 suggest that they regulate cell identity during olfactory system development.

Indeed, in the developing cerebral cortex Fezf2 plays an essential role in establishing projection neuron identity. In the cerebral cortex, Fezf2 is initially expressed in progenitor cells. When neurogenesis begins, Fezf2 expression is restricted to subcortical projection neurons and regulates their identities (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Hirata et al., 2006a; Molyneaux et al.,
In Fezf2−/− mice, subcerebral neurons fail to develop and instead adopt the identities of alternative projection neuron subtypes; they express molecular markers of callosal and corticothalamic neurons and project axons to the normal targets of callosal and corticothalamic neurons (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Molyneaux et al., 2005). Current literature supports the idea that Fezf2 promotes subcerebral neuron identity by repressing alternative callosal and corticothalamic fates (Chen et al., 2008).

The dynamic expression pattern of Fezf2 in the olfactory system is similar to that observed in the cerebral cortex. It is expressed in VNO precursor cells before E14.5. However, when supporting cells are generated, Fezf2 expression becomes restricted to the sustentacular cells and excluded from VNO neurons. Based on a similarly dynamic expression pattern for Fezf2 in the cerebral cortex and VNO, and the mechanisms of Fezf2 regulation of projection neuron identities in the cerebral cortex, we propose that Fezf2 promotes supporting cell identity by repressing expression of VSN enriched genes. Unfortunately, the early and rapid degeneration of the VNO in Fezf2−/− mice prevented us from directly investigating whether Fezf2 represses VNO sensory neuron identity in supporting cells.

Fezf1 is essential for the axons of OSNs to project into the olfactory bulb (Hirata et al., 2006b; Watanabe et al., 2009). In our study, we found that, in addition to regulating axonal development, Fezf1 regulates the development of OSNs in the MOE through repression of VSN-enriched genes. In Fezf1−/− mice, the expression of many MOE-enriched genes is reduced and we observed ectopic expression of many
VNO-enriched genes. These results demonstrate that by repressing VSN-enriched
gene expression, \textit{Fezf1} ensures that OSNs fully differentiate and mature. Since
olfactory axons do not reach the olfactory bulb in \textit{Fezf1}\textsuperscript{-/-} mice, possibly due to a
failure to penetrate the basal lamina layer of the brain (Watanabe \textit{et al.}, 2009), we
were unable to determine whether the target choice of \textit{Fezf1} mutant OSNs is switched
from the main olfactory bulb to the accessory olfactory bulb.

There are two identifiable protein domains in the FEZ proteins: an engrailed
homology domain and the zinc-finger motifs. Both protein domains are almost
identical between FEZF1 and FEZF2, suggesting that they can regulate common gene
targets. Indeed, we observed the same effects on axonal targeting when either FEZF1
or FEZF2 was mis-expressed in corticocortical projection neurons in the cerebral
cortex. Recently, Shimizu \textit{et al.} reported that FEZF1 and FEZF2 both directly repress
transcription of \textit{Hes5} (Shimizu \textit{et al.}, 2010a). This report and our results are
consistent with the hypothesis that \textit{Fezf1} and \textit{Fezf2} regulate cell fate during
development of the olfactory system through repression of VSN-enriched genes in
either progenitors and sensory neurons of the MOE or supporting cells of the VNO
(Figure 2-10M).

Our study helps to illuminate the molecular mechanisms that regulate the
contrasting identities of the MOE and VNO, two functionally and anatomically
distinct organs that arise from a common placode during development. Separate
olfactory subsystems first appeared in the aquatic ancestors of modern tetrapods, and
this subsystem organization has been maintained in primates (Swaney and Keverne,
We propose that Fezf1 and Fezf2 function to prevent OSNs in the MOE and supporting cells in the VNO from assuming a VNO sensory neuron identity. FEZF1 and FEZF2 likely accomplish this task either through activation or repression of target genes. The mixed cell identities observed in the MOE of Fezf1+/− mice suggests that, although Fezf1 plays an important role in repressing VSN identity, there are likely other key regulators that act either upstream or in parallel to promote OSN fate specification.

Materials and Methods

Generation of Fezf1−/− and Fezf1−/−; Fezf2−/− mice

A targeting vector was designed to replace the Fezf1 coding region with a cassette containing enhanced green fluorescent protein (EGFP) followed by an internal ribosome entry site (IRES)-human placental alkaline phosphatase (hPLAP) and PGK-neo (Figure 2-3A). It contains a 3 kb homologous sequence upstream of and including the sequence encoding the first 10 amino acids of FEZF1, a 5.2 kb EGFP-IRES-hPLAP cassette, a 2 kb PGK-neo cassette, a 4.3 kb homologous sequence downstream of the Fezf1 gene, and the RSV-TK negative selection cassette. The junction of Fezf1 and EGFP was sequenced to ensure that no mutations were generated during cloning and that the EGFP ORF was in frame. The linearized knockout construct was electroporated into E14a ES cells, which were subjected to both positive and negative selections. Correctly-targeted ES clones were identified by Southern hybridization. Two clones were used to generate chimeric mice by blastocyst injection. After germline transmission of the mutant allele, heterozygous
*Fezf1*\(^{+/−}\) mice were mated to β-actin-cre CD1 mice to excise the floxed pgk-neo selection cassette. *Fezf1*\(^{+/−}\) mice were then mated to generate *Fezf1*\(^{−/−}\) mice. Proper expression of the targeted allele was confirmed by *in situ* hybridization to detect hPLAP mRNA (Figure 2-3C, D).

Generation of *Fezf2*\(^{+/−}\) mice has been described previously (Chen *et al.*, 2005a). *Fezf1*\(^{+/−}\) mice were bred with *Fezf2*\(^{+/−}\) mice to obtain *Fezf1*\(^{+/−}\); *Fezf2*\(^{+/−}\) compound heterozygotes. Breeding *Fezf1*\(^{+/−}\); *Fezf2*\(^{+/−}\) mice with each other generated animals carrying various combinations of *Fezf1* and *Fezf2* mutant alleles.

Genotyping of *Fezf1* alleles was accomplished by PCR using two sets of primers. The wild type allele was genotyped using p1 (ATGGACAGTAGCTGCCTCAACGCGACC) and p2 (ATGTTCACGCTCGCAGCCGGTGACAC), with an expected PCR product of 589 bp (Figure 2-3A, B). The mutant *Fezf1* allele was genotyped using p3 (GGGGAGCTCTCTCAACATG) and p4 (GGGGAGCTCTCTCAAATG), yielding the expected product of 460 bp (Figure 2A, B). The PCR conditions were 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 64°C for 1 min and 72°C for 1 min. Genotyping of *Fezf2* alleles was reported previously (Chen *et al.*, 2005a).

The day of vaginal plug detection was designated as embryonic day 0.5 (E0.5). The day of birth was designated as postnatal day 0 (P0). Experiments were carried out in accordance with protocols approved by the IACUC at the University of California, Santa Cruz and were performed in accordance with institutional and federal guidelines.
Immunohistochemistry

Tissue samples were fixed overnight in 4% paraformaldehyde at 4°C and transferred to 30% sucrose overnight at 4°C. The following morning they were frozen in Tissue-Tek O.C.T. compound (Sakura Finetek). 20 µm tissue sections were collected onto Superfrost slides (Fisher Scientific). Alternatively, 50 µm floating sections were processed for immunohistochemistry. Primary antibodies used are described in Table 2-1. Primary antibodies were detected using AlexaFluor-conjugated secondary antibodies (Invitrogen, 1:1000). Nuclei in Figure 2 were visualized using SYTOX orange (Invitrogen, 1:100,000).

Immunohistochemistry was performed as follows. Tissue sections were washed three times for five minutes each in PBS (pH 7.4) and placed into blocking solution (10% horse serum, 0.3% Triton X-100 in PBS) for one hour at room temperature. Primary antibodies were added at the appropriate dilutions (Table 2-1) and incubated overnight at 4°C. The following day sections were washed three times in PBS and incubated with the secondary antibodies for two hours at room temperature. Sections were then washed three times in PBS and cover-slipped with Fluoromount-G (Southern Biotech).

Antibody Characterization

The antibodies used in this study are listed in Table 2-1. Characterization and controls for each primary antibody used are described below. The anti-cleaved caspase-3 antibody detects the large fragment (17/19 kDa) of caspase-3 resulting
Table 2-1

List of antibodies used for this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Source (Cat. No.)</th>
<th>Dilution</th>
<th>Immunogen</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaved</td>
<td>Rabbit (P)</td>
<td>Cell Signaling Technology, Danvers, MA (9661)</td>
<td>1:200</td>
<td>synthetic peptide (KLH-coupled) CRGTELDCTIEGTD adjacent to D175 in human caspase-3</td>
</tr>
<tr>
<td>Caspase-3</td>
<td></td>
<td></td>
<td></td>
<td>recombinant GFP protein emulsified in Freund’s adjuvant</td>
</tr>
<tr>
<td>GFP</td>
<td>Chicken (P)</td>
<td>Aves Labs, Tigard, OR (GFP-1020)</td>
<td>1:1000</td>
<td>c-terminal peptide between amino acids 377–394 of rat</td>
</tr>
<tr>
<td>Goαoff</td>
<td>Rabbit (P)</td>
<td>Santa Cruz Biotechnology, Santa Cruz, CA (sc-383)</td>
<td>1:25</td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>Rat (M)</td>
<td>Chemicon, Temecula, CA (MAB5272)</td>
<td>1:500</td>
<td>glycoprotein fraction from the cerebellum of C57BL/6J mice</td>
</tr>
<tr>
<td>NCAM</td>
<td>Rat (M)</td>
<td>Chemicon, Temecula, CA (MAB310)</td>
<td>1:500</td>
<td>glycoprotein fraction from neonatal mouse brain</td>
</tr>
<tr>
<td>OMP</td>
<td>Goat (P)</td>
<td>Wako, Richmond, VA (544-10001)</td>
<td>1:1000</td>
<td>rodent protein</td>
</tr>
<tr>
<td>PHH3</td>
<td>Mouse (M)</td>
<td>Cell Signaling Technology, Danvers, MA (9706)</td>
<td>1:100</td>
<td>synthetic phospho-peptide (KLH coupled) ATKQTARKSTGGKAKA surrounding S10 of human histone H3</td>
</tr>
</tbody>
</table>

P, polyclonal; M, monoclonal
from cleavage adjacent to D175 in western blot analysis of extracts from HeLa, NIH/3T3 and C6 cells. It does not recognize full-length caspase-3 or other cleaved caspases (manufacturer’s technical information). Immunostaining using this antibody detects apoptotic cells both in culture and in tissue sections. The specificity of the staining has been confirmed by the use of blocking peptide in both western analysis and in immunohistochemistry. Antibody preincubated with control peptide stained cells in mouse embryonic tissue or human tonsil tissue sections, but antibody preincubated with cleaved caspase-3 (Asp175) blocking peptide (Cell signaling, #1050) completely failed to stain mouse or human tissue sections. In addition, western blot analysis of cell extracts from Jukrat cells treated with 0.25 mg/ml cytochrome C (to induce apoptosis) using cleaved caspases-3 antibody showed specific bands at 17 and 19 kDa. Western blot analysis of the same cell extract using cleaved caspase-3 antibody preincubated with the blocking peptide (1/100) prevented the antibody from recognizing the 17/19 kDa bands (manufacturer’s technical information).

The anti-GFP antibody labels neurons only in mice carrying an EGFP targeted allele or transgene and not in wild type animals. This staining pattern recapitulates the intrinsic fluorescence of the EGFP.

The anti-Gαs/olf antibody was developed against a rat C-terminal peptide (aa 377-394) (Daniel Crews, Santa Cruz Biotech, personal communication). Western blotting of T98G or HeLa whole cell lysates recognizes 3 bands (52 kDa, 45 kDa, 39 kDa) corresponding to the long and short forms of Gαs and Gαolf (manufacturer’s
technical information). In our study, this antibody labeled the cilia of olfactory neurons, where olfactory receptors bind to odorant molecules and initiate cell signaling events. This staining pattern is consistent with the mRNA expression pattern of $G\alpha_{\text{s/olf}}$, which was detected in the olfactory neurons of the MOE by \textit{in situ} hybridization (Eckler and Chen, unpublished result). Our \textit{in situ} hybridization results showed that compared to wild type P0 mice, the expression level of $G\alpha_{\text{s/olf}}$ mRNA was decreased in the MOE of $\text{Fezf1}^{-/-}$ P0 mice. The reduced staining with anti-$G\alpha_{\text{s/olf}}$ antibody in the MOE of $\text{Fezf1}^{-/-}$ mice confirmed the specificity of this antibody.

The anti-L1 rat monoclonal antibody was generated by immunizing rats with a glycoprotein fraction from P8-P9 mouse cerebellum. The antibody reacts with both mouse and rat tissues in immunohistochemistry. In western blot analysis, it recognizes the 220-240 kDa mouse L1 protein (manufacturer’s technical information). The specificity of this antibody has been confirmed using L1 knockout mice. In lysates from mutant mice, western blot analysis showed a complete absence of the normal L1 protein. In wild type mice, L1 staining was detected in the ventral-lateral columns of spinal cord at e11.5 and in the dorsal root ganglion axons. In the L1 mutant mice, L1 staining was not detected (Cohen et al., 1998). In the olfactory system and in mouse brains, this antibody labeled the cell body and projections of neurons.

Specificity of the anti-NCAM antibody has been demonstrated by the use of NCAM knockout animals. In $\text{NCAM}^{-/-}$ mice no staining was observed with this antibody (Schellinck et al., 2004). We observed similar staining patterns to those
previously described (Inaki et al., 2002; Schellinck et al., 2004; Taniguchi et al., 2003), with strong labeling of neuronal cell bodies and projections.

The anti-OMP antibody strongly labels mature sensory neurons throughout the olfactory system (Marcucci et al., 2009; Watanabe et al., 2009). This antibody recognizes a single band of 19 kD by western blot using mouse brain extract (Baker et al., 1989). In tissue sections we observed strong labeling of cell bodies and axons of both OSNs and VSNs. This labeling pattern is consistent with the mRNA expression pattern of OMP detected by in situ hybridization (Eckler and Chen, unpublished result).

The anti-phosphorylated histone H3 (ser10) antibody detects endogenous levels of histone H3 only when phosphorylated at serine 10. This antibody does not cross-react with other phosphorylated histones or acetylated histone H3. The specificity of this antibody was confirmed using several assays. Western blot analysis of whole cell lysates from NIH/3T3 cells treated with serum plus calyculin A to induce histone H3 phosphorylation recognized a 16.5 kD band, which was not observed in untreated samples. Flow cytometric analysis of pacilitaxel-treated THP1 cells using this antibody versus Propidium Iodide showed that the cells stained by this antibody contained higher DNA content, corresponding to cells that passed S phase of the cell cycle. Co-staining cultured NIH/3T3 cells with the anti-phosphorylated histone H3 antibody with a tubulin antibody (to label microtubule cytoskeleton and mitotic spindles) showed that only dividing cells were stained by the anti-phosphorylated histone H3 antibody, and that nonmitotic cells were unstained. Immunohistochemical
analysis of paraffin-embedded human carcinoma tissues using this anti-phosphorylated histone H3 antibody showed specific staining, but the staining was abolished when the tissue was treated with phosphatase, or when the antibody was preincubated with 38phosphor-histone H3 (ser10) blocking peptide (#1000, Cell Signaling) (manufacturer’s technical information). In our hands, this antibody strongly labeled cells in the ventricular and subventricular zone in developing mouse brains that were undergoing mitosis (Betancourt and Chen, unpublished observation).

**PLAP staining**

PLAP activity was detected with AP staining buffer (0.1 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 1 mg/ml nitroblue tetrazolium in 100 mM Tris-HCl pH9.5, 100 mM NaCl) as previously described (Chen et al., 2008).

**In situ hybridization**

*In situ* hybridization using $^{35}$S-labeled probes was performed as previously described (Chen et al., 2005a). Fresh-frozen olfactory tissue was fixed in 4% paraformaldehyde for 20 minutes at room temperature and then dehydrated with ethanol. Slides were treated with proteinase K for 30 minutes at room temperature, acetylated for 10 minutes, and then washed with 2X SSC. $^{35}$S-labeled cRNA probes were hybridized to sections overnight at 65°C. The following day slides were washed with SSC and subjected to Rnase A digestion for 30 minutes at 37°C. Slides were then dehydrated and dipped in Kodak NTB2 emulsion 1:1 with distilled water. Kodak
D19 and Rapid Fix solutions were used to develop the slides and cresyl violet was used as a counter stain.

Non-radioactive in situ hybridization was performed essentially as described (Schaeren-Wiemers and Gerfin-Moser, 1993). Briefly, digoxigenin-labeled cRNA probes were synthesized for the genes of interest. Probes were hybridized to sections of fresh-frozen olfactory tissue overnight and visualized by the addition of NBT/BCIP substrate at room temperature.

Probes used in this study are described in Table 2-2. In addition to their primary targets, the following probes likely cross react with additional mRNAs based on their high degree of homology. The OR mix probe is 94% homologous to nucleotides 214-895 of Olfr1198 (NM_207567.1). The Taar mix probe is 98% homologous to nucleotides 3-863 of Taar8b (NM_001010837), 99% homologous to nucleotides 42-902 of Taar8c (NM_001010840), and 81% homologous to nucleotides 30-860 of Taar6 (NM_001010828.1). The V1R mix probe is 88% homologous to nucleotides 132-858 of V1rb1 (NM_053225.1), 90% homologous to nucleotides 132-858 of V1rb2 (NM_011911.1), 90% homologous to nucleotides 228-956 of V1rb3 (NM_053226.2), 91% homologous to nucleotides 130-858 of V1rb4 (NM_053227.2), 88% homologous to nucleotides 130-855 of V1rb8 (NM_053229.1), and 91% homologous to nucleotides 130-858 V1rb9 (NM_053230.1). The V2R mix probe is 99% homologous to nucleotides 1500-2405 of Vmn2r2 (NM_001104592.1), 93% homologous to nucleotides 1698-2591 of Vmn2r3 (NM_001104614.1), 94% homologous to nucleotides 1512-2405 of Vmn2r4 (NM_001104615.1), 96%
### Table 2-2

List of *in situ* probes used for this study

<table>
<thead>
<tr>
<th>Probe (type)</th>
<th>Target</th>
<th>Preperation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cnga2 (S)</td>
<td>nucleotides 17925 of mouse Cnga2 (*NC-000086)</td>
<td>primers (5’-GCGCGCAATTAACCTCTACTAAGGGAA TAGGAGACGCTGATGAAGGA A-3’) and (5’- GCGCGGTAATA CAGC-3’), incorporating T3 or T7 promoter sequences respectively, were used to generate a template for <em>in vitro</em> transcription with T7 polymerase</td>
</tr>
<tr>
<td>Fezfl (S)</td>
<td>nucleotides 289-828 of mouse Fezfl (*NC-000072)</td>
<td>primers (5’-GCGCGCCTAATACGACTCAGTATAGGGCATGGA CAGT AGCTGCTCAAACGGGACCA-3’) and (5’- GCGCGCAATTAACCCTC ACTAAGGGAACGGGATGGTTCACGCTCGATC-3’), incorporating T7 or T3 promoter sequences respectively, were used to generate a template for <em>in vitro</em> transcription with T3 polymerase</td>
</tr>
<tr>
<td>Fezfl (S)</td>
<td>nucleotides 644-1374 of mouse Fezfl (*NC-000080)</td>
<td>primers (5’-GCGCGCTAATAGCTAGCCATAGGGCATGCC CAGC TCAGCTTCCCTGGAGCATGG-3’) and (5’- GCGCGCAATTAACCCTCAGTATAGGGGAATTCCTC CTTCAAGCCCTGGTCACTC-3’), incorporating T7 or T3 promoter sequences respectively, were used to generate a template for <em>in vitro</em> transcription with T3 polymerase</td>
</tr>
<tr>
<td>5’OR mix (D)</td>
<td>nucleotides 212-895 of mouse Olf1 (Olf1208 (*NC_000068.6))</td>
<td>the PCR product from primers (5’-CTTCCACAGTAGCCCAAAA-3’) and (5’-AACCCTTCCATGGACATTCC-3’) was cloned into pGEM-T (Promega), linearized with Nco I, and transcribed with SP6 polymerase</td>
</tr>
<tr>
<td>5’TAAR mix (D)</td>
<td>nucleotides 3-863 of mouse Taar8a (*AC_000032.1)</td>
<td>the PCR product from primers (5’-GACCAGCAACTTTTCCCAAC-3’) and (5’-ATGAAGCCCATGAAAGCATC-3’) was cloned into pGEM-T (Promega), linearized with Nco I, and transcribed with SP6 polymerase</td>
</tr>
<tr>
<td>Trpe2 (S)</td>
<td>nucleotides 7985-8594 of mouse Trpe2 (*NC-000073)</td>
<td>primers (5’-GCGCGCAATTAACCTCTACTAAGGGGAACGC C AA CTTGGACTGAGATTGTAACAAA-3’) and (5’- GCGCGGTAATACGA CTTCAACTATGAGGTGGTGTGTCTGCGGATAGTG -3’), incorporating T3 or T7 promoter sequences respectively, were used to generate a template for <em>in vitro</em> transcription with T7 polymerase</td>
</tr>
</tbody>
</table>
Table 2-2 continued

<table>
<thead>
<tr>
<th>5VIR mix (D)</th>
<th>nucleotides</th>
<th>the PCR product from primers (5’-AGGCTTAAGCCCATGGATCT-3’) and (5’-AGGCTTGACTGTGGCATAGC-3’) was cloned into pGEM-T (Promega), linearized with Not I, and transcribed with T7 polymerase</th>
</tr>
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<tbody>
<tr>
<td>mouse Virb7</td>
<td>130-858</td>
<td></td>
</tr>
<tr>
<td>(*NC-000072.5)</td>
<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>5V2R mix (D)</th>
<th>nucleotides</th>
<th>the PCR product from primers (5’-ACCAGGACAGAGGAATGTG-3’) and (5’-TCAACAGTGAGGACTCTCC-3’) was cloned into pGEM-T (Promega), linearized with Neo I, and transcribed with SP6 polymerase</th>
</tr>
</thead>
<tbody>
<tr>
<td>mouse Vmm2r1</td>
<td>22833-23738</td>
<td></td>
</tr>
<tr>
<td>(*NC-000069)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

D, digoxigenin (DIG)-labeled probe; S, 35S-labeled probe; *GenBank accession number; 5See materials and methods for additional targets
homologous to nucleotides 1500-2405 of \(Vmn2r5\) (NM_001104618.1), 93% homologous to nucleotides 1512-2405 of \(Vmn2r6\) (NM_001104619.1), and 93% homologous to nucleotides 1513-2405 of \(Vmn2r7\) (NM_175674.3).

**Microscopy**

Confocal images were captured on a Leica TCS SP5 confocal microscope. Bright and dark field images were captured on an Olympus BX51 microscope using a Q Imaging Retiga Exj camera. Images were processed using Adobe Photoshop CS4 to adjust brightness and contrast.

**Cell death and proliferation analysis**

E12.5 tissue sections were processed for immunohistochemistry with mouse anti-phosphorylated histone H3 and rabbit anti-cleaved caspase-3 antibodies. Total numbers of immunoreactive cells were counted on alternating 20 \(\mu\)m tissue sections for \(Fezf2^{+/+}\) and \(Fezf2^{-/-}\) VNOs. Total phosphorylated histone H3\(^{+}\) and cleaved caspase-3\(^{+}\) cells were averaged for each genotype and the standard error of mean (SEM) calculated. P-values were generated using a Student’s \(t\)-test and statistical significance was assigned to p-values of \(\leq 0.05\).

**Isolation of Fezf1-EGFP\(^{+}\) cells by FACS**

The main olfactory epithelium from E18.5 \(Fezf1^{+/+}\) or \(Fezf1^{-/-}\) animals was dissected in cold Neurobasal (NB) solution (Invitrogen) which had been equilibrated
overnight at 37°C in a tissue culture incubator and then placed on ice. Dissections were performed using a fluorescence dissecting microscope to ensure that VNO tissues were not harvested. Tissue from mice of identical genotypes from the same litter were pooled and dissociated by incubation in a papain solution (Worthington) for 30 minutes in a 32°C water bath. Enzymatic activity was quenched by washing with trypsin inhibitor and BSA, and tissues were subjected to gentle trituration. Dissociated cells were resuspended in cold PBS with 2% BSA and filtered through a 100 µm filter. *Fezf1*-EGFP<sup>+</sup> cells were isolated by FACS on a BD Biosciences Aria cell sorter. Propidium iodide staining was used to exclude dead cells.

**Microarray analysis**

Isolated E18.5 *Fezf1*-GFP<sup>+</sup> cells were immediately processed for total RNA isolation using an Rneasy Micro Kit (Qiagen). RNA samples from *Fezf1<sup>+/−</sup>* and *Fezf1<sup>−/−</sup>* were reverse transcribed, labeled and hybridized to Affymetrix Mouse Gene 1.0 ST Arrays. Microarray experiments were performed at the Stanford Protein and Nucleic Acid (PAN) facility. Expression signals were normalized and the gene list was generated using the Partek Genomics Suite. Briefly, signal intensities for triplicate *Fezf1<sup>+/−</sup>* or *Fezf1<sup>−/−</sup>* samples were combined into a single expression score and converted to log<sub>2</sub> scale. Pairwise analysis was performed and transcript level changes with a fold enrichment of 1.75 or greater and an ANOVA-generated p-value of 0.05 or less were considered significant. All microarray data have been deposited in the Gene Expression Omnibus database at NCBI (Accession GSE26064).
**In utero electroporation**

EGFP or Fezf1 cDNA was cloned into the pCMV expression vector. EGFP or EGFP plus Fezf1 plasmids were electroporated into the cerebral cortex of E15.5 CD-1 embryos as described (Chen et al., 2008). Animals were allowed to develop until P5 and axons were visualized by staining with EGFP antibodies.
Chapter 3: *Fezf2* expression identifies a multipotent progenitor for neocortical projection neurons, astrocytes and oligodendrocytes

Introduction

The cerebral cortex contains six layers of projection neurons and glia. Projection neurons in each cortical layer display similar morphologies, axonal projections and gene expression patterns (Kwan *et al.*, 2012). During development, cortical projection neurons are generated from radial glial cells (RGCs) and basal progenitors in an inside-out pattern such that deep-layer neurons are generated first, followed by upper-layer neurons (Molyneaux *et al.*, 2007). Three decades of work based upon transplantation experiments (Desai and McConnell, 2000; McConnell, 1985; McConnell and Kaznowski, 1991), viral lineage tracing (Luskin *et al.*, 1988; Walsh and Cepko, 1988) and *in vitro* culture of single early or late RGCs (Shen *et al.*, 2006) suggests that cortical projection neuron subtype is sequentially determined by birthdate through progressive lineage restriction of a common RGC (Leone *et al.*, 2008). However, the identification of early CUX2-expressing (CUX2+) RGCs, which were reported to be intrinsically specified to generate late-born, upper-layer neurons (Franco *et al.*, 2012), calls into question this decades-old model and raises the possibility that deep-layer projection neurons are similarly generated from lineage-restricted RGCs (Franco and Muller, 2013; Marin, 2012).

The transcription factor *Fezf2* (also known as *Fezl* and *Zfp312*) is expressed in early cortical progenitors and deep-layer neurons and is critical for the proper fate-
specification of subcerebral projection neurons (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). In the Fezf2−/− mice, subcerebral projections are absent and deep-layer neurons instead switch their identity to become corticothalamic or callosal projection neurons (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Han et al., 2011; McKenna et al., 2011; Molyneaux et al., 2005). Several studies suggest that ectopic expression of Fezf2 in late cortical progenitors (Chen et al., 2008) or immature neurons (De la Rossa et al., 2013; Rouaux and Arlotta, 2012) redirects these cells to differentiate into subcerebral projection neurons. These results indicate that expression of Fezf2 in cortical progenitors may be sufficient to confer a subcerebral neuron identity, and thus Fezf2+ cortical progenitor cells may be lineage-restricted to generate deep-layer neurons (Franco and Muller, 2013; Woodworth et al., 2012).

To investigate the lineage potential of Fezf2-expressing progenitor cells we performed in vivo genetic fate mapping using the Fezf2 locus. Here we show that Fezf2+ cortical progenitor cells are RGCs that exist throughout cortical neurogenesis. Temporal fate mapping demonstrated that Fezf2+ RGCs sequentially generated projection neuron subtypes and glia based upon the birthdate of these cells. Further, Fezf2+ RGCs generated upper-layer neurons without transitioning though a CUX2+ RGC stage. Finally we demonstrate that CUX2+ cells in the embryonic ventricular and subventricular zones are not RGCs but rather immature interneurons migrating from the subcortical telencephalon. Collectively these results indicate that Fezf2+ RGCs are a multipotent progenitor for neocortical projection neurons, astrocytes and
oligodendrocytes and suggest that progressive lineage restriction of a common RGC is the predominant mechanism for generating neuronal diversity in the mammalian cerebral cortex.

Results

Lineage traced Fezf2-expressing progenitor cells are RGCs

We first characterized Fezf2 expression by in situ hybridization. As previously reported (Hirata et al., 2004), we detected Fezf2 expression in early neocortical progenitors (Figure 3-1D and data not shown). Interestingly, Fezf2 expression in the ventricular zone (VZ) persisted postnatally, long after deep-layer neuron generation has ceased (Figure 3-1A). We confirmed this by investigating GFP expression in Fezf2-GFP BAC transgenic mice (Gong et al., 2003; Shim et al., 2012), which revealed that GFP+ cells persist in the VZ during late embryonic and early postnatal stages (Figure 3-1B). To assess the differentiation potential of Fezf2+ progenitor cells, we generated nine independent Fezf2-CreERT2 BAC transgenic mouse lines (Figure 3-1C). In situ hybridization for Cre and Fezf2 showed that Cre expression was identical to that of endogenous Fezf2 (Figure 3-1D-G). Breeding these mice to three different Cre reporter lines (RCE-GFP, R26R-LacZ or TauR-mGFP) (Friedrich and Soriano, 1991; Hippenmeyer et al., 2005; Sousa et al., 2009) revealed that the fused CreERT2 protein was tightly regulated by tamoxifen (Figure 3-2A-C). Although Cre mRNA was expressed in deep-layer neurons (Figure 3-1E, G), we observed tamoxifen-induced recombination in these neurons only using the TauR-mGFP
The Fezf2-CreER<sup>T2</sup> allele facilitates temporal fate-mapping of neocortical progenitor cells. (A) In situ hybridization shows Fezf2 expression in the VZ of P0 wild-type brains (black arrow). (B) GFP-expressing cells are present in the VZ of P0 Fezf2-GFP BAC transgenic mice (white arrow). (C) Strategy for generation of Fezf2-CreER<sup>T2</sup> mice. (D-G) In situ hybridization for Fezf2 (D, F) and Cre (E, G) at E13.5 (D-E) and P7 (F-G) demonstrates that Cre recapitulates Fezf2 expression. (H, H') GFP<sup>+</sup> cells in the brains of P21 mice that received tamoxifen at E18.5. (H') An enlarged view of the boxed area in (H). (H') All GFP<sup>+</sup> cells displayed astrocyte or oligodendrocyte morphology, however GFP<sup>+</sup> projection neurons in layer 5 were not present, demonstrating the lack of recombination in postmitotic neurons. Ctx, cerebral cortex; LV, lateral ventricle; Thal, thalamus; VZ, ventricular zone; WM, white matter. Scale bars: 250 μm.
Characterization of *Fezf2-CreER<sup>T2</sup>* mice. (A–B') No recombination was observed in *Fezf2-CreER<sup>T2</sup>; RCE-GFP (A–A') or *Fezf2-CreER<sup>T2</sup>; R26R-LacZ (B–B') animals after vehical administration. (C) Recombination in *Fezf2-CreER<sup>T2</sup>; R26R-LacZ mice after tamoxifen administration. (D-E) Recombination was observed in deep-layer postmitotic neurons of *Fezf2-CreER<sup>T2</sup> TauR-mGFP mice. Ctx, cerebral cortex; Hip, hippocampus; Str, striatum; Thal, thalamus. Scale bars: (A, B) 500 μm, (A', B') 250 μm, (C) 100 μm, (D) 50 μm, (E) 25 μm.
reporter (Figure 3-2D-E). No recombination was observed in postmitotic neurons upon tamoxifen administration with the Rosa26R-LacZ or RCE-GFP reporter alleles (Figure 3-1H-H’ and Figure 3-2C). Critically, this allowed us to perform lineage-tracing experiments for Fezf2+ cortical progenitor cells using the RCE-GFP reporter without the ambiguity caused by Cre-mediated recombination in postmitotic neurons.

Examination of Fezf2-CreER\textsuperscript{T2}; RCE-GFP mice after tamoxifen induction revealed that recombination specifically marked Fezf2+ RGCs (Figure 3-3). Twenty-four hours after tamoxifen administration, approximately 80% of GFP\textsuperscript{+} cells expressed the RGC marker SOX2, while approximately 10% of GFP\textsuperscript{+} cells expressed the basal progenitor marker TBR2 (Figure 3-3A-B, D-E, J). The majority of GFP\textsuperscript{+} cells were located in the VZ; many had both apical and basal processes and divided at the ventricular surface (Figure 3-3D-F), all of which are characteristic of RGCs. The few TBR2\textsuperscript{+}GFP\textsuperscript{+} cells were likely basal progenitors recently generated from Fezf2+ RGCs. Supporting this, three days after an E13.5 tamoxifen administration (TM @ E13.5; E16.5), the fraction of TBR2\textsuperscript{+}GFP\textsuperscript{+} cells in the VZ and SVZ increased to 39% (Figure 3-3G-J). These results indicate that lineage-traced Fezf2+ progenitors are RGCs.

Fezf2+ RGCs sequentially generate deep-layer and upper-layer cortical projection neurons and glia

To assess the lineage potential of Fezf2+ RGCs, we administered tamoxifen to Fezf2-CreER\textsuperscript{T2}; RCE-GFP mice at different embryonic stages. In mice that received
**Fezf2-expressing progenitors are RGCs.** (A-C) Low magnification images of GFP+ cells in the cortex of Fezf2-CreER<sup>2</sup>; RCE-GFP mice following CRE-mediated recombination. (D-F) 24 hours after tamoxifen induction, most GFP+ cells expressed SOX2 (78 ± 3%) (D), and few cells expressed TBR2 (10 ± 2%) (E). (F) GFP+ cells dividing at the ventricular surface. (G-I) In TM @ E13.5; E16.5, Fezf2+ RGCs gave rise to basal progenitors. (G) GFP was expressed in both VZ and SVZ progenitors (arrowhead), migrating neurons in the intermediate zone (arrow), and cortical neurons (asterisk). (H) GFP+ cells expressed SOX2 (54% ± 3) and showed typical RGC morphology. (I) Many GFP+ cells expressed TBR2 (39% ± 2). (J) Quantification of the percentage of GFP+SOX2+ RGCs and GFP+TBR2+ basal progenitors among all the GFP+ cells in the VZ and SVZ ± SEM. * P < 0.05, ** P < 0.005, *** P < 0.0001. CP, cortical plate; Ctx, cerebral cortex; IZ, intermediate zone; LV, lateral ventricle; SVZ, sub-ventricular zone; VZ, ventricular zone. Scale bars: (A, B, C) 250 μm (B insert) 10 μm, (E, F, G, I) 25 μm.
tamoxifen at E12.5 and were examined at P21 (TM @ E12; P21), GFP+ cells were detected throughout the cortical plate (Figure 3-4A-B) and included cortical projection neurons (75%) and glia (25%) (Figure 3-4A-F, Q). GFP+ neurons were present in both deep (36%) and upper (64%) cortical layers (Figure 3-4A-D, L). Those in layer 5 expressed high-levels of CTIP2, indicating a subcerebral neuron identity (Figure 3-4C). Many GFP+ cells expressed SATB2, a marker for callosal projection neurons, and were observed in both deep- and upper-layers (Figure 3-4D). Indeed, GFP labeled axon tracts demonstrated that Fezf2+ RGCs generated callosal, subcerebral and corticothalamic projection neurons (Figure 3-5). In addition to projection neurons, 15% of GFP+ cells showed an astrocytic morphology and expressed GFAP (Figure 3-4E, Q) while 10% of GFP+ cells expressed the oligodendrocyte marker OLIG2 (Figure 3-4F, Q). In brains that contained putative RGC clones, the GFP+ cells within the clones consisted of neurons in both deep and upper layers, astrocytes and oligodendrocytes (Figure 3-4R). These results demonstrate that at E12.5, Fezf2+ RGCs are not lineage-restricted; instead they are multipotent, producing deep- and upper-layer neocortical neurons, astrocytes and oligodendrocytes.

In TM @ E14.5; P21 brains, GFP+ cells included projection neurons, astrocytes and oligodendrocytes (Figure 3-4G-L, Q). However, the majority (94%) of GFP+ neurons were present in upper layers, and many expressed high levels of SATB2 (Figure 3-4G-I). Retrograde tracing from the contralateral cortical hemisphere showed that GFP’SATB2+ cells projected callosal axons (Figure 3-6). In
Fezf2+ RGCs are multi-potent, and become progressively restricted as development proceeds. (A–F) Immunohistochemical analysis of TM @ E12.5; P21 brains. (A, B) GFP+ cells were present throughout the cortex. GFP+ cells expressed CTIP2 (C), SATB2 (D), GFAP (E) or OLIG2 (F). (G–K) Immunohistochemistry on brain sections from TM @ E14.5; P21 mice. (G, H) GFP+ cells expressed SATB2 (I), GFAP (J), or OLIG2 (K). (L) Percentage of GFP+ neurons in deep- and upper-layers ± SEM. (M–P) Immunohistochemical analysis of brains from TM @ E18.5; P21 mice. GFP+ cells included astrocytes (O) and oligodendrocytes (P). (Q) Percentage of GFP+ cells that were neurons, astrocytes or oligodendrocytes ± SEM. (R) Clonal analysis of single RGCs indicates that early Fezf2-expressing RGCs can generate deep- and upper-layer neurons, astrocytes and oligodendrocytes. * P < 0.05, ** P < 0.005, *** P < 0.0001. Ctx, cerebral cortex; Hip, hippocampus; Thal, thalamus; WM, white matter. Scale bars: (A, G, M) 500 μm, (B, H, N) 250 μm, (F, K, P, R4) 25 μm, (R) 100 μm.
Early Fezf2+ RGCs generate all major projection neuron subtypes. Fezf2+ RGCs generate callosal projections (A), corticothalamic projections (B) and corticospinal projections (C). Scale bar: (C) 500 μm.
**Figure 3-6**

**Mid Fezf2⁺ RGCs generate callosal projection neurons.** (A) Whole mount brain indicating injection site of Ctβ into the corpus callosum. (B-I) Upper-layer Ctβ traced neurons originating from Fezf2⁺ RGCs labeled with tamoxifen at E14.5, project to the contralateral hemisphere (B, D-F) and express SATB2 (C, G-I). Ctx, cerebral cortex; OB, olfactory bulb. Scale bars: (A) 100 μm, (C, I) 200 μm.
TM @ E18.5; P21 brains only 2% of GFP+ cells were projection neurons. Instead, the GFP+ cells consisted of astrocytes (53%) and oligodendrocytes (45%) (Fig. 2-4M-Q). We observed similar results when we examined lineage-traced brains at P8 (data not shown). Collectively, these results indicate that Fezf2+ RGCs are multipotent and become progressively lineage-restricted to generate neocortical projection neurons and glia according to their birthdate.

**Fezf2+ RGCs generate CUX2+ upper-layer neurons without transiting through a CUX2+ RGC stage**

The finding that Fezf2+ RGCs contribute substantially to upper layer neurogenesis is consistent with the classic progressive restriction model (Leone et al., 2008). However, this is in contrast to a newly proposed model that suggests all upper-layer neurons are generated from Cux2+ RGCs (Franco and Muller, 2013). To resolve this difference, we explored the relationship between Fezf2+ RGCs and Cux2+ cortical cells using two different CUX2 antibodies (Conforto et al., 2012; Iulianella et al., 2008; Laz et al., 2007). We found that 72% of upper-layer neurons generated from Fezf2+ RGCs expressed CUX2 (Figure 3-7A-B). Since Cux2+ projection neurons were reported to arise from Cux2+ RGCs (Franco et al., 2012), we next investigated whether Fezf2+ RGCs transit through a CUX2+ RGC stage to generate upper-layer neurons. In agreement with previous reports (Cobos et al., 2006; Cubelos et al., 2008a; Cubelos et al., 2008b; Franco et al., 2012; Franco et al., 2011; Nieto et al., 2004; Zimmer et al., 2004), we detected robust CUX2 expression beginning at
**Figure 3-7**

Fezf2⁺ RGCs generate CUX2⁺ upper-layer neurons without transitioning through a CUX2⁺ RGC stage. (A-B) Fezf2⁺ RGCs generated CUX2⁺ upper-layer neurons. (C-E') Immunohistochemical analysis of TM @ E10.5; E15.5 brains. (C-C') CUX2⁺ cells were detected in the VZ, SVZ and CP however; the majority of CUX2⁺ cells were in the SVZ and CP. (D-D') Few CUX2⁺ cells in the SVZ expressed SOX2 and these cells were located at the VZ/SVZ boundary. (E-E') Rare GFP⁺ cells in the VZ/SVZ expressed CUX2, but the GFP⁺CUX2⁺ cells did not express SOX2. The arrowheads in C'-E' point to the CUX2⁺SOX2⁺ cells in VZ/SVZ. The arrows in C'-E' point to a rare GFP⁺CUX2⁺ cell. (F-J) Dlx1/2-Cre; Ai14 and Nkx2.1-Cre; Ai14 mice demonstrate that the majority of CUX2⁺ cells in the E15.5 neocortical VZ and SVZ were interneurons generated from the Dlx1/2 (F-G) or Nkx2.1 (H-I) lineage. (J) Quantification of the percentage of CUX2⁺TdTomato⁺ cells among all CUX2⁺ cells in the VZ and SVZ ± SEM. CP, cortical plate; IZ, intermediate zone; SVZ, subventricular zone; VZ, ventricular zone. Scale Bars: (B) 500 μm, (E, E', G, I) 25 μm.
E14.5, including expression in neocortical interneurons (Figure 3-8). We examined the relationship between Fezf2\(^+\) RGCs and CUX2\(^+\) progenitors using TM @ E10.5; E15.5 Fezf2-CreER\(^T2\); RCE-GFP brains, since the Cux2\(^+\) RGC population was previously reported to peak at this age (Franco et al., 2012). We found that while some CUX2\(^+\) cells resided in the VZ, the majority were in the SVZ and cortical plate (Figure 4C-C’). Within the VZ/SVZ, very few CUX2\(^+\) cells expressed the RGC marker SOX2 (Figure 3-7D-D’). The few SOX2\(^-\)CUX2\(^+\) cells were located in the SVZ or the interface between the VZ and SVZ (Figure 3-7D-D’). We rarely observed GFP\(^-\)CUX2\(^+\) cells in the VZ/SVZ, and these cells were SOX2\(^-\), suggesting they were not RGCs, but were likely basal progenitors generated from Fezf2\(^+\) RGCs (Figure 3-7E’-E’). Taken together, these results indicate that Fezf2\(^+\) RGCs generate upper-layer neurons without transiting through a CUX2\(^+\) RGC stage.

**CUX2\(^+\) cells in the neocortical VZ/SVZ are immature interneurons, not RGCs**

The lack of GFP\(^-\)CUX2\(^+\) RGCs in the VZ/SVZ of TM @ E10.5; E15.5 brains prompted us to investigate the lineage origin of CUX2\(^+\) cells in the neocortical VZ/SVZ. Since CUX2 was previously reported to be expressed in interneurons (Cobos et al., 2006; Cubelos et al., 2008a; Cubelos et al., 2008b; Franco et al., 2012; Franco et al., 2011; Zimmer et al., 2004), we determined the percentage of CUX2\(^+\) cells in the E15.5 neocortical VZ/SVZ that originated from the subcortical telencephalon. DLX1 and DLX2, two transcription factors required for the proper development of cortical interneurons, are expressed in the lateral, medial and caudal
**Figure 3-8**

**CUX2 expression during early- to mid-corticogenesis.** (A-C) At E10.5 we detected robust CUX2 expression within the cortical hem. (D-F) At E10.5 we did not detect CUX2 expression in the neocortex. (G-I) At E13.5 CUX2 was mainly expressed in the marginal zone. (J) CUX2 was detectable at E14.5 throughout the cortex. However the majority of CUX2⁺ cells were in the SVZ and cortical plate and exhibited a nuclear morphology reminiscent of migrating interneurons. (K-M) Many CUX2⁺ cells in the VZ, SVZ and cortical plate expressed the interneuron marker GAD67. Ctx, cerebral cortex; CH, cortical hem; CP, cortical plate; LV, lateral ventricle; PP, preplate; VZ, ventricular zone. Scale Bars: (A) 250 μm, (C, F, I) 25 μm, (J) 50 μm, (M) 25 μm.
ganglionic eminences (Anderson et al., 1997; Long et al., 2009a; Long et al., 2009b; McKinsey et al., 2013). NKX2.1 is a homeobox transcription factor that is expressed in the developing medial ganglionic eminence and is required for interneuron production (Butt et al., 2008; Sussel et al., 1999). To label subcortically-derived neocortical interneurons at E15.5, we crossed either the Dlx1/2-Cre (Potter et al., 2009), or the Nkx2.1-Cre (Xu et al., 2008) alleles to mice harboring the Ai14 (TdTomato) (Madisen et al., 2010) Cre-dependent reporter. In these mice, cortical interneurons generated from the ventral forebrain were labeled by TdTomato expression (Figure 3-7G, I). Examination of E15.5 Dlx1/2-Cre; Ai14 or Nkx2.1-Cre; Ai14 brains revealed that the vast majority (94% or 98% respectativly) of CUX2+ cells in the neocortical VZ/SVZ were TdTomato+ (Figure 3-7F-J). This result demonstrates that in the developing neocortex, nearly all CUX2+ cells in the VZ/SVZ are immature interneurons derived from the subcortical telencephalon. Thus CUX2+ cells are unlikely to make a significant contribution to the generation of CUX2+ upper-layer cortical projection neurons.

Discussion

Our lineage analysis demonstrates that both early and late Fezf2+ RGCs are multipotent. Early Fezf2+ RGCs generate all cortical projection neuron subtypes as well as astrocytes and oligodendrocytes. Late Fezf2+ RGCs generate upper-layer neurons, astrocytes and oligodendroctes. Thus their potential is progressively restricted in accordance with the classic model of cortical neurogenesis (Leone et al.,
2008). However, our results do not exclude the existence of intrinsically fate-restricted RGCs outside of a common lineage. Rather, the finding that many CUX2\(^{+}\) upper-layer neurons arise from the Fezf2-lineage without transitioning through a CUX2\(^{+}\) RGC stage, combined with the few CUX2\(^{+}\) RGCs in the VZ, suggests that much of neocortical neurogenesis is accomplished through a multipotent RGC.

A comparison between \textit{Fezf2-CreER}\(^{T2}\) mice and the \textit{Cux2-Cre} and \textit{Cux2-CreER}\(^{T2}\) mice used by Franco et al. reveals the underlying reasons for the drastically different conclusions reached by these two studies. Upon tamoxifen administration, the \textit{Fezf2-CreER}\(^{T2}\) allele induces recombination of the \textit{RCE-GFP} reporter only in progenitor cells and not in postmitotic neurons. However, in the Franco et al. study, the \textit{Cux2-Cre} and \textit{Cux2-CreER}\(^{T2}\) alleles induced recombination of the \textit{Ai9} reporter in both progenitor cells and postmitotic neurons (Franco \textit{et al.}, 2012; Franco \textit{et al.}, 2011) masking the true lineage potential of Cux2\(^{+}\) progenitor cells. Further, \textit{Fezf2} is not expressed in neocortical interneurons or their progenitors. In contrast, \textit{Cux2} is expressed at high levels in interneurons that are born in the subpallium and migrate into the neocortex (Cobos \textit{et al.}, 2006). The Franco et al. study did not consider this possibility for the origin of the Cux2\(^{+}\) cells in the neocortical VZ/SVZ.

Our results indicate that during cortical development, the \textit{Fezf2} locus is under dynamic transcriptional regulation. Twenty-four hours after tamoxifen administration to \textit{Fezf2-CreER}\(^{T2}\); \textit{RCE-GFP} embryos, the majority of lineage-traced cells were RGCs. Since \textit{Fezf2} is expressed in deep-layer neurons immediately after their birth (Figure 1D), this indicates that during cortical neurogenesis \textit{Fezf2} is expressed in
RGCs but is rapidly silenced in basal progenitors similar to genes such as Sox2 and Pax6. However, in deep-layer cortical projection neurons Fezf2 expression is turned on again, immediately after these neurons are born. Similar dynamic expression patterns have been observed for other genes that are essential for cortical development, including Foxg1 (Miyoshi and Fishell, 2012) and Nfib (Betancourt and Chen, unpublished result). Identifying the cis-regulatory sequences (Visel et al., 2013) that differentially promote Fezf2 expression in RGCs vs. postmitotic neurons, as well as the trans-factors that bind these sequences (Shim et al., 2012), will provide insight into the mechanism by which Fezf2+ RGCs generate both Fezf2-expressing deep-layer neurons, as well as upper-layer neurons that do not express Fezf2.

Previous studies suggest that neocortical RGCs are a heterogeneous cell population. Subsets of RGCs were reported to differentially express markers such as RC2, GLAST and BLAST (Hartfuss et al., 2001). In addition, in vivo lineage analysis of neocortical progenitors using retroviral vectors produced clones that consisted of only neurons or glia (Luskin et al., 1988; Walsh and Cepko, 1988), suggesting the existence of neuron- or glia-restricted progenitor cells. Further, recent work indicates that the neocortical progenitor pool contains both outer radial glia cells (Hansen et al., 2010; Wang et al., 2011) as well as short neural precursors (Gal et al., 2006; Stancik et al., 2010; Tyler and Haydar, 2013) that are both molecularly and morphologically distinct from ventricular zone RGCs .

Results from our study indicate that progressive lineage restriction of multipotent RGCs is a critical mechanism for generating cellular diversity during
development of the cerebral cortex. However, the finding that CUX2\(^+\) cells in the neocortical VZ/SVZ are unlikely to make a significant contribution to the generation of upper-layer cortical projection neurons does not exclude the possibility that some cortical projection neurons may originate from lineage-restricted RGCs. Many genes expressed in RGCs have been identified (Molyneaux et al., 2007; Woodworth et al., 2012). Genetic fate mapping using these loci will further expand our knowledge of progenitor cell heterogeneity within the neocortical progenitor pool and uncover the mechanisms that generate projection neuron diversity during development of the cerebral cortex.

**Material and Methods**

**Mice**

All experiments were carried out in accordance with protocols approved by the IACUC at University of California at Santa Cruz, and were performed in accordance with institutional and federal guidelines. Generation of *RCE-GFP* (Sousa et al., 2009), *R26R-LacZ* (Friedrich and Soriano, 1991), *tauR-mGFP* (Hippenmeyer et al., 2005), *Ai14* (Madisen et al., 2010), *Dlx1/2-Cre* (Potter et al., 2009) and *Nkx2.1-Cre* (Xu et al., 2008) mice was previously described. *Fezf2-CreER\(^T2\)* mice were generated according to previously established strategies (Lee et al., 2001) by modifying the RP23-141E17 BAC. The modified BAC was purified and injected into zygotes derived from FVB mice according to established protocols (Gong and Yang, 2005).
Fezf2-CreERT2 mice were generated by modifying the RP23-141E17 BAC, which contains the mouse Fezf2 gene plus 36 kb of 5' flanking sequence and 160 kb of 3' flanking sequence was targeted with the pERFN vector containing a CreERT2-frt-neo-frt cassette flanked by approximately 500 bp homology A and B boxes. CreERT2 was inserted in front of the Fezf2 open reading frame through homologous recombination by a defective prophage system. The start codon of the Fezf2 ORF was mutated to prevent expression of FEZF2 from the transgene. The A box was amplified from RP23-141E17 BAC by PCR using primers 5’-GGTACCCCTTGGGTGCACTTTGTTCTG-3’ and 5’-GGCGCGCCAAGCTGGGCCAGGCTGGGC-3’. The B box was amplified using primers 5’-GTTTAAACGCCAGCTCAGCTTCCCTGGAG-3’ and 5’-GCGGCCGCGACGACACAGCCACAGTTGGT-3’. The modified BAC was purified and injected into zygotes derived from FVB mice according to established protocols (Gong and Yang, 2005).

Tamoxifen induction of Fezf2-CreERT2 lines was achieved by single intraperitoneal injection (4-6mg/40g body weight) of tamoxifen (Sigma) dissolved in corn oil into pregnant mothers at indicated gestation stages. Alternatively, for high efficiency labeling 1mg/40g body weight of 4-hydroxytamoxifen (Sigma) dissolved in 100% EtOH was injected intraperitoneally.

The day on which a vaginal plug was detected was designated as embryonic day 0.5 (E0.5). The day of birth was designated as postnatal day 0 (P0).
Identification of single RGC clones

Clonal analysis was performed on TM @ E12.5; P21 Fezf2-CreERT2; RCE-GFP brains, using low-efficiency tamoxifen induction. GFP+ cell bodies in one cortical hemisphere were present in 2 or 3 neighboring sections. Based on the low number of cells labeled (average 10 cells/brain), and the proximity of the cells, we identified these cells as the progeny from a single RGC.

Histology

In situ hybridization for Fezf2 and Cre was performed as described (Eckler et al., 2011). The Cre probe was generated by PCR using primers 5’-TGCCACGACCAAGTGACAGCAATG-3’ and 5’-ACCAGAGACGGAAATCCATCGCTC-3’. Immunohistochemistry was performed as previously described (McKenna et al., 2011). The rabbit anti-CUX2 antibody (Conforto et al., 2012; Laz et al., 2007) was a gift from Dr. David Waxman. We also confirmed CUX2 expression using a second rabbit anti-CUX2 antibody (Iulianella et al., 2008; Iulianella et al., 2009) kindly provided by Angelo Iulianella. The following additional primary antibodies were used: chicken anti-LACZ (Abcam ab9361), goat anti-SOX2 (Santa Cruz Biotech sc-17320), rabbit anti-TBR2 (Abcam ab23345), rat anti-CTIP2 (Abcam ab18465), rabbit anti-SATB2 (Abcam ab34735), chicken anti-GFP (Aves GFP-1020), rabbit anti-GFAP (Dako Z0334), rabbit anti-OLIG2 (Abcam ab33427), rabbit anti-S100β (Dako Z0311), and mouse anti-GAD67 (Millipore MAB5406). Fluorescent secondary antibodies were from Jackson laboratories. Nuclei
were visualized with DAPI (Molecular Probes). For cell counting a minimum of 3 sections were counted from 3 experimental replicates. To determine statistical significance, GraphPad was used to preform an unpaired t-test between experimental groups.

**Retrograde Tracing**

Retrograde tracing was performed using AlexaFluor™ 594-conjugated Cholera Toxin β subunit (CT-β) (Invitrogen) injections into one cortical hemisphere of P1 mice. The brains were collected at P21 for analysis.
Chapter 4: Multiple conserved regulatory domains promote Fezf2 expression in the developing cerebral cortex

Introduction

Current estimates suggest that greater than half of evolutionarily conserved sequences in the human genome do not correspond to protein coding regions (Lander et al., 2001; Mouse Genome Sequencing et al., 2002; Siepel et al., 2005; Venter et al., 2001). Transcriptional enhancers constitute a substantial portion of these regions (Williamson et al., 2011). Genome wide association studies (GWAS) have identified sequence variations within putative enhancers that are associated with a wide range of neurological disorders such as autism, epilepsy and schizophrenia (Genomes Project, 2010; Hamilton et al., 2005; International Schizophrenia, 2008; Sebat et al., 2007; Wray, 2007). However, the functional relevance of these DNA elements to brain development is poorly understood (Visel et al., 2009).

The cerebral cortex contains six layers of projection neurons that are generated in a stereotyped manner such that deep-layer neurons, layer (L) 5 and 6, are born before upper-layer neurons (Kwan et al., 2012). We, and others, have previously shown that the transcription factor Fezf2 is necessary and sufficient for generation of L5 corticospinal motor neurons (CSMNs) (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Molyneaux et al., 2005). These studies indicate that Fezf2 functions at the top of a transcriptional hierarchy that regulates CSMN development and fate specification (Chen et al., 2008; De la Rossa et al., 2013; Rouaux and
Arlotta, 2010; Rouaux and Arlotta, 2012). Despite the essential role of Fezf2 in neural development, little is known about how its transcription is precisely regulated.

To uncover the regulatory mechanisms controlling Fezf2 transcription, we utilized chromatin immunoprecipitation combined with high throughput DNA sequencing (ChIP-seq) to identify a transcription factor-binding signature around the Fezf2 locus. Guided by our ChIP-seq data, we mapped a promoter sufficient for Fezf2 expression in the cortex, and investigated the activity of two putative Fezf2 enhancers. We demonstrate that enhancer 434 is sufficient to drive expression in cortical progenitor cells while enhancer 1316 is strictly active in deep-layer projection neurons. Taken together, these results illustrate how distinct cis-regulatory elements can specify temporal and spatial patterns of gene expression during nervous system development.

Results

Identification of cis-regulatory elements near the Fezf2 locus

To understand how Fezf2 transcription is regulated, we closely examined its expression during cortical neurogenesis (Figure 4-1A-C’). At E12.5 Fezf2 expression was detected in ventricular zone (VZ) progenitors and deep-layer cortical neurons (Figure 4-1A-A’). At E15.5 Fezf2 expression began to decline in L6, but remained high in L5 (Figure 4-1B-B’). Expression at P0 was similar to E15.5 (Figure 4-1C-C’). Thus, at late stages of cortical development, expression of Fezf2 was restricted to three distinct domains: higher expression in L5 neurons, and lower expression in L6
**Figure 4-1**

**Fezf2 expression is dynamically regulated.** *In situ* hybridization at E12.5 (A-A’), E15.5 (B-B’), and P0 (C-C’). (D) Diagram of the Fezf2 locus indicating positions of the 2.7 kb promoter and the putative enhancers 434, and 1316. (E-F) ChIP-seq was used to map the binding of transcription factors to the Fezf2 locus. (E) An overview of binding around enhancer 434 and the upstream conserved region. (F) An overview of factor binding around enhancer 1316. (E-F) Peaks called by IDR are highlighted in red. Peaks called by MACS are highlighted in blue. Cxx, cortex; LGE, lateral ganglionic eminence; LV, lateral ventricle; MGE, medial ganglionic eminence; PP, preplate; Str, striatum; VZ, ventricular zone. Scale bars: (A) 100 μm, (A’) 10 μm, (B) 250 μm, (B’) 25 μm. (C) 500 μm, (C’) 50 μm.
neurons and progenitors (Figure 4-1A-C’). This dynamic spatial and temporal expression pattern suggests that \textit{Fezf2} is under the control of a complex regulatory program.

Recent work has identified transcription factors that are essential for cortical neuron fate specification and differentiation. Among these, SOX5 and TBR1 appear to directly regulate \textit{Fezf2} (Han \textit{et al.}, 2011; Kwan \textit{et al.}, 2008; McKenna \textit{et al.}, 2011). Using chromatin isolated from E15.5 mouse cortices, we mapped the binding of transcription factors previously shown to be important for cortical development. These included FOXG1, NFIB, SATB2, SOX5, SOX6 and TBR1 (Alcamo \textit{et al.}, 2008; Azim \textit{et al.}, 2009; Baranek \textit{et al.}, 2012; Batista-Brito \textit{et al.}, 2009; Britanova \textit{et al.}, 2008; Han \textit{et al.}, 2011; Hanashima \textit{et al.}, 2004; Kwan \textit{et al.}, 2008; Lai \textit{et al.}, 2008; McKenna \textit{et al.}, 2011; Miyoshi and Fishell, 2012; Piper \textit{et al.}, 2009). We found that FOXG1, SOX5 and TBR1 bound to the evolutionarily conserved region immediately upstream of \textit{Fezf2} (Figure 4-1E), suggesting this region may contain the \textit{Fezf2} promoter. Interestingly, all of these factors, with the exception of SOX6, bound to a previously identified enhancer downstream of \textit{Fezf2}, enhancer 434 (Figure 4-1E). In addition to the upstream region and enhancer 434, FOXG1, SATB2 and TBR1 bound a region in the neighboring \textit{Cadps} gene, near the 5’ end of \textit{Fezf2} (Figure 4-1F).

Taken together, our ChIP-seq results indicate that multiple transcription factors that are critical for cortical development bind at or near the \textit{Fezf2} locus.

To determine the significance of our ChIP-seq data we searched the VISTA Enhancer Browser (www.enhancer.lbl.gov) for sequences in and near \textit{Fezf2} that may
regulate its expression (Visel et al., 2007). This approach uncovered several enhancers around Fezf2. These included an enhancer at the 5’ side of Fezf2, enhancer 1316, and an enhancer at the 3’ side, enhancer 434 (Figure 4-1D). Our ChIP-seq data demonstrated strong binding within or near these enhancers, suggesting they may be important for regulating Fezf2 within the cerebral cortex (Figure 4-1E-F).

Mapping the Fezf2 minimal promoter

Previously we identified the 2.7 kb region upstream of and including the Fezf2 start codon as sufficient to drive forebrain expression before the onset of cortical neurogenesis (Hirata et al., 2006a). This region was bound by multiple transcription factors in our ChIP-seq (Figure 4-1E). To study its activity throughout cortical development, we generated stable transgenic lines expressing the 2.7 kb-LacZ transgene. At E11.5, when the earliest-born cortical projection neurons were being generated, we observed LacZ activity throughout the cortex that mimicked endogenous Fezf2 expression (compare Figure 4-2A with Figure 4-1A). We observed β-gal expression in SOX2+ cortical progenitor cells and in TBR1+ and Tuj1+ postmitotic neurons (Figure 4-2B-D).

We next examined 2.7 kb promoter activity at P0. Whole mount LacZ staining revealed expression throughout the cerebral cortex in a high anterior-medial to low posterior-lateral pattern (Figure 4-2E). However, unlike endogenous Fezf2, which is expressed in the VZ and in deep cortical layers (L5 and L6), the 2.7 kb-LacZ transgene was active in all cortical layers (compare Figure 4-2F with Figure 4-1C).
Figure 4-2

Promoter activity of the 2.7 kb fragment during cortical development. (A) LacZ staining at E11.5 revealed reporter activity in the cerebral cortex, similar to endogenous Fez1f2 expression. (B-D) Immunohistochemistry with LacZ antibody combined with SOX2 (B), Tuj1 (C) and TBR1 (D) antibodies. (E) Whole mount LacZ staining of P0 brains showed extensive expression in the forebrain. (F) LacZ staining of P0 brain sections. (G-I') Immunohistochemistry using LacZ antibody combined with antibodies for SOX2 (G, G'), CTIP2 (H, H') and SATB2 (I, I'). Arrows represent co-expression of LacZ and the indicated marker. Arrowheads indicate a lack of co-expression. PP, preplate. Scale bars: (A) 200 μm, (D) 50 μm, (E) 250 mm, (F) 500 μm, (I) 100 μm, (I') 50 μm.
LacZ was expressed in SOX2\(^+\) progenitor cells in the VZ and in the CTIP2\(^+\) or SATB2\(^+\) projection neurons (Figure 4-2G-I’). LacZ expression at P12 was similar to that observed at P0 (Figure 4-3). Taken together, these results indicate that the 2.7 kb promoter was active throughout cortical development and was sufficient to drive transcription across all endogenous *Fezf2* expression domains. However, its expression in upper cortical layers suggested that additional *cis*-elements are required to properly coordinate *Fezf2* expression.

**Isolation of a *Fezf2* forebrain enhancer**

Next, we defined the sequences within the 2.7kb promoter that were necessary for *Fezf2* forebrain expression during early development using transient transgenic mice (Figure 4-4). Deletion of the 5’ 1.5 kb from the 2.7 kb fragment (1.2 kb-\(\text{LacZ}\)) resulted in a complete loss of LacZ expression in forebrain at E8.5, even though LacZ activity was consistently observed in the tail (Figure 4-4A-B). These results suggest the presence of a forebrain enhancer located between 2.7 kb and 1.2 kb upstream of the translation start codon.

By adding back sequences to the 1.2 kb promoter region, we mapped the *Fezf2* forebrain enhancer to a 50 bp region upstream of the 1.2 kb fragment (Figure 4-4C-I). Sequence analysis demonstrated this region to be highly conserved across vertebrates (Figure 4-5A). When we cloned 8x arrays of this 50 bp region into the 1.2 kb reporter construct (1.2 kb-FB-50 bp-\(\text{LacZ}\)), we observed reporter expression in the forebrain (Figure 4-5B-C). We then cloned 8x arrays of either the 3’ 27 bp or 35 bp of
**Figure 4-3**

2.7 kb promoter activity at P12. (A) The activity of the 2.7 kb promoter at P12 was similar to that observed at P0, with expression throughout the cortex and the strongest activity in upper layers. (B-C') LacZ was expressed in some CTIP2$^+$ and TBR1$^+$ cells in deep layers. (D-D') However, the highest density of LacZ$^+$ cells was in upper layers cells that expressed SATB2. B'-D' showes amplified areas boxed in panels B-D, respectively. Arrows represent co-expression of LacZ and the indicated marker. Arrowheads indicate a lack of co-expression. Ctx, cortex; LV, lateral ventricle; Str, striatum. Scale bars: (a) 500 µm, (d) 100 µm, (d') 50 µm.
Identification of the *Fezf2* forebrain enhancer. (A) An overview of the successive truncations used to determine that the 1.2 kb-LacZ reporter construct is not active in the E8.5 forebrain. (B) Whole mount LacZ staining of E8.5 1.2 kb-LacZ embryos. (C-H) To further map the *Fezf2* forebrain enhancer, we added back a 200 basepair (bp) sequence present in the 2.7 kb fragment that was deleted in the 1.2 kb fragment (C). Mice expressing this construct demonstrated abundant reporter activity in the forebrain, indicating that the *Fezf2* forebrain enhancer is contained within this region (D). We generated constructs containing 8x arrays of either the proximal or the distal 150 bp of this region (E). Transgenic mice expressing either of these constructs reliably exhibited reporter activity in the forebrain similar to the 2.7 kb-LacZ reporter construct, indicating that the middle 100 bp of this region contained the *Fezf2* forebrain enhancer. (H) Focusing on this 100 bp region, we generated embryos expressing LacZ under control of this sequence plus the 1.2 kb promoter fragment (1.2 kb-FB(medial100 bp)-LacZ). (I) Embryos harboring this reporter construct expressed LacZ in the forebrain. (J) Further truncation of the forebrain enhancer indicated that the 3' 50 bp was sufficient to drive LacZ expression in the forebrain of E8.5 embryos. Scale bar: (I) 1 mm.
Mapping of the *Fezf2* forebrain enhancer. (A) An overview of the region corresponding to the *Fezf2* forebrain enhancer. The expanded sequence shows a Multiz alignment demonstrating the high level of vertebrate conservation of this enhancer. (B) The reporter construct used to dissect the activity of this enhancer. 8 x arrays of either the entire 50 bp enhancer or the 3’ 35 or 27 bp sequences were cloned upstream of the 1.2 kb promoter which lacks forebrain activity. (C-E) Whole mount LacZ staining indicating that the 3’ 35 bp region is required for the forebrain activity of this enhancer. Scale bar: (E) 1 mm.
the forebrain enhancer into the 1.2 kb reporter construct (Figure 4-5B, D-E). LacZ activity was observed in the forebrain of mice harboring the 1.2 kb-FB-35 bp-LacZ but not the 1.2 kb-FB-27 bp-LacZ construct (Figure 4-5D-E), demonstrating that the Fezf2 forebrain enhancer was contained within this 35 bp sequence. Taken together, these results indicate that within the 2.7 kb promoter is a 35 bp enhancer that is both necessary and sufficient for Fezf2 expression in the forebrain.

Enhancer 434 is active in forebrain progenitors

Previous work has shown that enhancer 434 is highly conserved across vertebrates and is necessary for Fezf2 expression in the cortex (Shim et al., 2012). Deletion of this enhancer from either a bacteria artificial chromosome containing a Fezf2-EGFP reporter or from its endogenous locus resulted in a loss of EGFP or Fezf2 expression in the cerebral cortex (Shim et al., 2012). However, it remains unknown whether enhancer 434 alone is sufficient to promote Fezf2 expression in subcerebral neurons.

We generated stable transgenic lines expressing LacZ under control of enhancer 434 and the hsp68 minimal promoter. At E11.5, β-gal was expressed in SOX2+ and PAX6+ cortical progenitor cells (Figure 4-6A-B), and in metaphase cells that were labeled by PHH3 antibody at the ventricular surface (Figure 4-6C). Few Tuj1+ neurons expressed β-gal (Figure 4-6D).

Whole mount staining of P0 brains revealed strong LacZ activity throughout multiple brain regions (Figure 4-6E). However, the majority of enhancer 434 activity
Enhancer 434 is active in cortical progenitor cells. (A-C) Immunohistochemistry demonstrated LacZ expression in progenitor cells at E11.5 that co-expressed SOX2 (A), PAX6 (B) and PHH3 (C). LacZ was also expressed in a few post-mitotic neurons in the preplate marked by Tuj1 (D). LacZ staining of whole mount P0 brains (E) and sections (F) demonstrated reporter activity in the VZ. (G-H) Immunohistochemistry using LacZ antibody combined with antibodies for RGC markers BLBP (G) and PAX6 (H). (I, K-L) In adult animals, the majority of enhancer 434-LacZ⁺ cells were in GFAP⁺ astrocytes throughout the forebrain. A subset of non-astrocyte like cells expressed SATB2 (J-J'). Arrows represent co-expression of LacZ and the indicated markers. Hip, hippocampus; WM, white matter. Scale bars: (D) 50 μm, (E) 250 mm, (F) 500 μm, (G) 25 μm, (H) 10 μm (I) 500 μm (J) 100 μm (J') 50 μm (K) 25 μm.
was in the VZ of the cortex and basal ganglia (Figure 4-6F). Immunohistochemistry with antibodies for LacZ and the radial glial cell (RGC) marker BLBP showed significant co-localization (Figure 4-6G). Additionally, some LacZ+ cells in the VZ expressed the RGC marker PAX6 (Figure 4-6H). Very few cells in the cortical plate expressed LacZ, and a minority of these LacZ+ cells expressed the projection neuron markers SOX5 and SATB2 (Figure 4-7A-B’). Thus, at P0 the majority of enhancer 434 activity was in progenitor cells.

In adult mice, LacZ expression was observed in the cerebral cortex, white matter, hippocampus, and striatum (Figure 4-6I-L). Most of the LacZ+ cells exhibited typical astrocyte-like morphology and expressed the astrocyte marker GFAP (Figure 4-6K-L). A minority of LacZ+ cells in the cerebral cortex expressed the cortical projection neuron marker SATB2 (Figure 4-6J’). Taken together, these data demonstrate that enhancer 434 was primarily active in progenitor cells, one of the endogenous expression domains of Fezf2.

**Enhancer 1316 is active in deep-layer postmitotic neurons**

We next investigated the activity of enhancer 1316. MultiZ alignment of this region showed strong vertebrate conservation, especially within the middle 500 bp (Figure 4-8). We generated stable transgenic lines expressing LacZ under control of enhancer 1316 and the hsp68 minimal promoter. At E11.5, β-gal was present throughout the newly generated preplate (Figure 4-9A), in Tuj1+ neurons (Figure 4-9D). LacZ+ cells did not express the progenitor markers PAX6 or SOX2 (Figure 4-
Figure 4-7

Enhancer 434 activity at P0. (A-B') LacZ expression was observed in a few post-mitotic neurons expressing SOX5 (A, A') or SATB2 (B, B'). A' and B' show amplified areas boxed in panels A and B. Arrows represent co-expression of LacZ and the indicated markers. Arrowheads indicate a lack of co-expression. Scale bars: (B) 100 μm, (B’) 50 μm.
**Vertebrate conservation of enhancer 1316.** The UCSC genome browser was used to perform a Multiz alignment of enhancer 1316 from 30 vertebrates. Strong conservation was most evident within the middle ~500 bp of this region. Basewise conservation is represented as greyscale darkness with higher conservation corresponding to darker values. Gap Annotation: single line, no bases in the aligned species; double line, aligning species has one or more un-alignable bases in the gap region; red coloring, aligning species has Ns in the gap region. Genomic Breaks: green square brackets, enclose shorter alignments consisting of DNA from one genomic context in the aligned species nested inside a larger chain of alignments from a different genomic context.
**Enhancer 1316 is active in deep layer cortical neurons.** (A-D) Immunohistochemical analysis of E11.5 brain sections using antibodies for LacZ (A), LacZ and PAX6 (B), LacZ and SOX2 (C), and LacZ and Tuj1 (D). (E-F) LacZ staining of P0 whole mount brains (F) and brain sections (F). (G-I) Immunohistochemistry using LacZ antibody combined with antibodies for SOX2 (G), CTIP2 (H), and SATB2 (I). (J) A model of Fezf2 regulation in the cortex. Arrows represent co-expression of LacZ and the indicated marker. Arrowheads indicate a lack of co-expression. Scale bars: (A) 200 μm, (D) 50 μm, (E) 250 mm, (F) 500 μm, (I) 100 μm, (I') 25 μm.
Whole mount staining at P0 demonstrated LacZ activity in deep layers of the cerebral cortex (Figure 4-9E). LacZ was expressed in the subplate and in some deep-layer neurons, but not in cortical progenitors or astrocytes (Figure 4-9F). Some LacZ+ cells expressed CTIP2 and SATB2 (Figure 4-9H-I’). None of the LacZ+ cells expressed SOX2 (Figure 4-9G, G’). Analysis at P12 revealed a similar LacZ expression pattern to that observed at P0 (Figure 4-10). Taken together, these results indicate that enhancer 1316 is strictly active in deep-layer neurons of the cerebral cortex.

**Discussion**

We set out to understand how non-coding DNA elements regulate nervous system development. Previous work indicates that Fezf2 transcription levels are critically important for specification of cortical projection neuron fate (Chen et al., 2005a; Chen et al., 2008; Chen et al., 2005b; Han et al., 2011; Kwan et al., 2008; Lai et al., 2008; McKenna et al., 2011; Molyneaux et al., 2005; Rouaux and Arlotta, 2010). Our ChIP-seq data indicated that multiple transcription factors bound evolutionarily conserved sequences around the Fezf2 locus. The large number of transcription factors that bound enhancer 434 underscores its developmental importance. Recent deletion analysis demonstrated that enhancer 434 was critical for corticospinal motor neuron identity and connectivity (Shim et al., 2012). In this study, the majority of enhancer 434 activity during cortical neurogenesis was in progenitor cells, suggesting that this element also functions to promote Fezf2 expression in the
Figure 4-10

Activity of Enhancer 1316 at P12. (A-D') Expression at P12 mirrored P0. Some LacZ positive cells in deep layers expressed CTIP2 (B-B'), SATB2 (C-C') and TBR1 (D-D'). B'-D' show enlargement of areas boxed in panels B-D. Ctx, cortex; LV, lateral ventricle; Str, striatum. Scale bars: (A) 500 μm, (D) 100 μm, (D') 20 μm.
VZ, one of the endogenous Fezf2 expression domains (Figure 4-6). Further, the binding of multiple transcription factors to this enhancer indicates that Fezf2 transcription is likely under the complex regulation of multiple transcription factors in a context-dependent manner.

Multiple cis-regulatory sequences around Fezf2 showed distinct yet overlapping activities during cortical development. These results are similar to the regulation of the eve gene locus in Drosophila where multiple, partially redundant enhancers control expression in individual stripes along the anterior-posterior axis of the embryo (Andrioli et al., 2002; Perry et al., 2011). Ultimately, combined activity of these enhancers is required to drive the precise spatio-temporal expression of eve. Similar regulatory paradigms have been uncovered in mammals through studying the interferon-beta enhancer (Thanos and Maniatis, 1995).

Given recent progress identifying putative mammalian enhancers (Visel et al., 2013), it is critical that we understand the spatial and temporal activities of these sequences in the regulation of individual genes, in order to appreciate the deleterious effects of mutations in non-coding sequences identified through GWAS. Wow! You made it to the end. Email me (matthew.eckler@gmail.com) for your free beer.

Focusing on Fezf2, a key regulator of cortical development and deep-layer projection neuron fate, our study demonstrates how multiple enhancer and promoter activities can coordinate the expression of a single locus during brain development. We propose that the combined activities of the 2.7 kb promoter and enhancers 434 and 1316 constitute a cis-regulatory module that controls Fezf2 in distinct domains of the
cerebral cortex, ultimately leading to its dynamic expression during cortical development (Figure 4-9J).

Materials and Methods

Animals

All experiments were carried out in accordance with protocols approved by the IACUC at the University of California, Santa Cruz and were performed in accordance with institutional and federal guidelines. The day of vaginal plug detection was designated as embryonic day 0.5 (E0.5). The day of birth was designated as postnatal day 0 (P0). Transgenic mice were generated using standard protocols (Nagy, 2003). For transient transgenic experiments, embryos were harvested at E8.5 and processed for LacZ staining. For stable transgenic lines, founders were outcrossed to CD1 wildtype females for a minimum of 3 generations before analysis. Number of founder lines generated: 2.7 kb-LacZ (2), enhancer 434-LacZ (3), enhancer 1316-LacZ (4).

Plasmids

Generation of the 2.7 kb-lacZ plasmid was previously described (Hirata et al., 2006a). The forebrain enhancer was mapped by connecting fragments with the 1.2 kb–LacZ construct, which was constructed by inserting the PstI(4)–NcoI fragment of Fezf2 promoter into the LacZ-pA plasmid.

For enhancer 434-lacZ, the genomic region was obtained from mouse genomic DNA by PCR using primers:
ATCGCTCGAGCAGGCTGTAGGATGGGCAGCAGGAGTTTC and ATCGAAGCTTGTAACAAGTCAGGTGAGCAGGCGGTA. The product was then cloned into the hsp68-lacZ expression vector using Gateway cloning according to the manufacture’s protocol (Visel et al., 2009).

Enhancer 1316-lacZ was generated from human genomic DNA using primers AAACCACACAGCTGGTTTCC and TTTCCGATAGATCGTCAGC and cloned into the hsp68-lacZ expression vector (Visel et al., 2013).

**Histology**

Immunostaining and in situ hybridization were performed as previously described (Eckler et al., 2011). The Fezf2 cRNA probe corresponds to nucleotides 644-1374 of mouse Fezf2 (GenBank: NC_000080). LacZ staining was performed according to standard protocols. Primary antibodies used: Chicken anti β-gal (Abcam, 1:500), Rabbit anti BLBP (Millipore, 1:500), Rat anti CTIP2 (Abcam, 1:1000), Guinea Pig anti GFAP (Advanced Immuno Chemical, 1:100), Rabbit anti PAX6 (Covance, 1:100), PHH3 (Cell Signaling, 1:100), Rabbit anti SATB2 (Abcam, 1:1000), Goat anti SOX2 (Santa Cruz Biotech, 1:500), Rabbit anti SOX5 (Abcam, 1:500), Rabbit anti TBR1 (Abcam, 1:1000), Mouse anti Tuj1 (Covance, 1:1000). Primary antibodies were detected using AlexaFluor-conjugated secondary antibodies (Invitrogen, 1:1000). DNA was visualized with DAPI (1:50,000).

**Microscopy**
Bright field and epiflorescence images were captured on an Olympus BX51 microscope using a Q Imaging Retiga Exj camera or a Keyence BZ-9000 microscope. Confocal images were captured on a Leica TCS SP5 confocal microscope. Images were processed using Adobe Photoshop CS5 to adjust brightness and contrast.

**ChIP-Seq**

Chromatin immunoprecipitation was preformed as previously described (McKenna et al., 2011). Antibodies used were Rabbit anti FOXG1 (Cell Signaling), Rabbit anti NFIB (Active Motif), Rabbit anti SATB2 (Abcam), Rabbit anti SOX5 (Abcam), Rabbit anti SOX6 (Abcam), Rabbit anti TBR1 (Abcam). Sequencing libraries were generated using the Illumina TruSeq kit according to the manufacturers protocol. Sequencing was preformed on an Illumina HiSeq 2000 at the UCSC Genome Technology Center. Input DNA was sequenced as control. Sequencing reads were mapped to the mouse genome (mm9) using the Bowtie mapping algorithm. Non-overlapping reads and PCR duplicates were removed. Peaks were called using both MACS and IDR algorithms (Li et al., 2011; Zhang et al., 2008)
Chapter 5: Conclusions

In the preceding chapters I described a series of experiments centered on the roles of Fez family transcription factors during the development of the mammalian nervous system. This body of work highlights the broad functions of these transcription factors in development of both the PNS and CNS and point to the critical function of Fezf1 and Fezf2 in specification of neuronal cell fates during embryogenesis. The future implications for this work will be discussed below.

The murine olfactory system as a model to study neuronal fate specification

The work described in chapter 2 demonstrates the complimentary functions of Fezf1 and Fezf2 in PNS development. During development of the olfactory system Fezf1 and Fezf2 display distinct and overlapping expression patterns. Fezf1 is expressed strongly in the MOE and more transiently in the VNO. In contrast Fezf2 is expressed strongly and specifically and in progenitors and sustentacular cells of the VNO. These expression patterns, combined with the mixed cellular identity of the MOE observed in Fezf1 mutant mice, suggests that these factors function, in part, to redundantly inhibit a VSN cell fate in MOE sensory neurons and VNO supporting cells respectively. Recent work on Fezf1 and Fezf2 function during early development of the cerebral cortex supports the hypothesis that a common function of these factors is to redundantly regulate that transcription of an overlapping set of target genes (Shimizu et al., 2010b). During early development of the cerebral cortex, FEZF1 and
FEZF2 both bind and repress the transcription of Hes5. Loss of either Fezf1 or Fezf2 has minimal effects on early cortical neurogenesis. However, in Fezf1; Fezf2 mutant mice neurogenesis is severely decreased and basal progenitor cell numbers are reduced (Shimizu et al., 2010b).

The fact that both of these studies reached a similar conclusion regarding the redundant function Fezf1 and Fezf2 indicates that the mouse olfactory system is a good model for the study of neuronal fate specification. Numerous genes that are important for cell fate specification during cerebral cortex development are also expressed in the olfactory system including Ctip2, Foxg1, Tbr1 and Satb2 (Enomoto et al., 2011). Although mutations in these genes all confer defects in cortical development and neuronal fate specification, their mechanisms of action are largely unknown. The reduced number of cell fate decisions that occur during olfactory system development (i.e. MOE vs. VNO), enable this tissue to serve as a simple model by which to probe the molecular mechanisms by which these factors regulate cell fate choices. In the future it will be interesting to screen for the expression of additional genes that are important for cortical development and cell fate specification within the olfactory system.

**Generation of cortical projection neuron subtypes**

Just as Fezf1 and Fezf2 control cell fate specification in the olfactory system, Fezf2 is also critical for specifying the identity of layer-five corticospinal motor neurons (Chen et al., 2005a; Chen et al., 2005b; Molyneaux et al., 2005). However,
Fezf2 is also expressed in neocortical progenitor cells. Whether Fezf2 functions in progenitor cells or postmitotic neurons to specify CSMN fate has remained unclear. The finding that Fezf2-expressing progenitor cells can generate all major classes of projection neurons and glia indicates that expression of Fezf2 in neural progenitors is not sufficient to generate CSMNs and suggests that Fezf2 is likely acting postmitotically in the promotion of a CSMN fate.

This result also has important implications for the burgeoning field of postmitotic reprogramming of cortical neuron subtypes. Several recent studies suggest that misexpression of Fezf2 in forebrain progenitors (Chen et al., 2008; Rouaux and Arlotta, 2010) or immature neurons (De la Rossa et al., 2013; Rouaux and Arlotta, 2012) can reprogram these cells into subcerebral projection neurons. However these experiments universally rely on in utero electroporation to express high levels of Fezf2 from an extrachromosomal plasmid. While the ability to redirect axons to subcortical regions remains consistent across all these studies, the molecular changes associated with the reported reprogramming are highly inconsistent. Initial reports indicated that only the axonal projections of the reprogrammed neurons were altered (Chen et al., 2008). However, more recent studies report the induction of genes normally associated with CSMNs including Ctip2, Er81, Sox5 and Tbr1 (De la Rossa et al., 2013; Rouaux and Arlotta, 2010; Rouaux and Arlotta, 2012). The discrepancies between these results may partially be explained by physical remolding of the brain during in utero electroporation based gene delivery. Going forward it will be critical
to repeat these experiments using less invasive methods for misexpression of Fezf2 such as inducible transgenic strategies.

Finally, the result that progressive lineage restriction of a common progenitor is the predominant mechanism for generating cellular diversity during cortical development has important implications for directing future research. It is likely that differential programs of gene expression may mark early versus late RGCs as their lineage potential becomes restricted. Refocusing efforts on the identification and function of these factors will help to uncover the mechanisms by which fate specification is temporally controlled during development of the cerebral cortex.

**Genome organization and neuronal fate specification**

The complex regulation of Fezf2 transcription by multiple cis-regulatory elements and trans-acting factors highlights the importance of genome organization during neuronal development. Chromosomal organization within the nucleus profoundly affects gene expression programs (Raab and Kamakaka, 2010). However in the context of cell fate specification, little is known about the role that chromosomal organization plays. One exception is olfactory receptor (OR) choice during OSN development. Recent work has demonstrated that silenced OR genes cluster in a few small loci around the nuclear periphery, at sites of heterochromatin (Clowney et al., 2012). Ultimately, this is thought to underly the unique expression of a single OR from approximately 1200 different OR genes.
A similar mechanism has also been reported during *Drosophila* neuroblast differentiation. The transcription factor Hunchback (Hb) is expressed in early neuroblasts and is necessary and sufficient for the generation of neurons born during an early (neuroblast divisions 1-5) competence window (Isshiki *et al.*, 2001; Novotny *et al.*, 2002). Initially during this competence window, the *hb* locus is located in the center of the nucleus where it is actively transcribed. However towards the end of the competence window the *hb* locus translocates to the nuclear periphery where its transcription becomes silenced (Kohwi *et al.*, 2013).

These two examples underscore the importance of genome organization to neuronal development. Going forward it will be important to extend these studies to cell fate specification both to help understand the effects of disease associated mutations in non-coding DNA regions as well as to advance potential therapeutics related to lineage reprograming.

**Implications for regenerative therapies**

One exciting application of this work is in regenerative medicine. There is much hope and promise surrounding stem cell replacement therapies for the treatment of diseases such as Parkinson’s and Alzheimer’s. However, for these strategies to be successful, it is imperative to efficiently and specify generate the distinct neuronal subtypes that are lost in these disorders. In order to accomplish this goal we must understand the basic developmental programs that govern their development and specification. Only then will it be possible to efficiently derive these cells types in
The integral role of Fezf1 and Fezf2 in neural fate specification during embryogenesis indicates that these factors may provide a molecular handle, both in the differentiation of embryonic stem cells, as well as for postmitotic reprogramming of mature neurons within diseased brains.
Bibliography


Baranek, C., Dittrich, M., Parthasarathy, S., Bonnon, C. G., Britanova, O., Lanshakov, D., Boukhtouche, F., Sommer, J. E., Colmenares, C., Tarabykin,


discrimination and male-male aggression in mice deficient for TRP2. *Science*,
295, 1493-1500.

homeobox gene function results in a ventral to dorsal molecular
respecification within the basal telencephalon: evidence for a transformation
of the pallidum into the striatum. *Development*, 126, 3359-3370.


Tanaka, H., Yamashita, T., Asada, M., Mizutani, S., Yoshikawa, H. and Tohyama, M.
(2002). Cytoplasmic p21(Cip1/WAF1) regulates neurite remodeling by

Taniguchi, M., Nagao, H., Takahashi, Y. K., Yamaguchi, M., Mitsui, S., Yagi, T.,
Mori, K. and Shimizu, T. (2003). Distorted odor maps in the olfactory bulb of

expression requires the assembly of an enhanceosome. *Cell*, 83, 1091-1100.

Tsuchida, T., Ensini, M., Morton, S. B., Baldassare, M., Edlund, T., Jessell, T. M. and

Tyler, W. A. and Haydar, T. F. (2013). Multiplex Genetic Fate Mapping Reveals a
Novel Route of Neocortical Neurogenesis, Which Is Altered in the Ts65Dn


