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FEZF2'S ROLE IN DIFFERENTIATION AND PROLIFERATION IN RADIAL GLIAL CELLS DURING CORTICAL DEVELOPMENT

A thesis submitted in partial
satisfaction of the requirements for the
degree of

MASTER OF SCIENCE

in

MOLECULAR, CELL, AND DEVELOPMENTAL BIOLOGY

by

Liora Huebner

December 2020

The Thesis of Liora Huebner is approved:

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Acting Vice Provost and Dean of Graduate Studies

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2020

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Abstract

FEZF2'S ROLE IN DIFFERENTIATION AND PROLIFERATION IN RADIAL GLIAL CELLS DURING CORTICAL DEVELOPMENT

Liora Huebner

Radial glial cells (RGCs) are multipotent progenitors that give rise to excitatory projection neurons, OB interneurons, oligodendrocytes and astrocytes in a temporarily and spatially regulated manner. The transcription factor FEZF2 is necessary for cortical deep layer neuron specification in postmitotic neurons. However, the function of FEZF2 in regulating proliferation and differentiation of RGCs during cortical development and into adulthood is poorly understood. Temporally regulated lineage tracing of RGC clones in the *Fezf2 KO* and *WT* shows that an absence of FEZF2 did not substantially impact the generation of glia. Similarly, when *Fezf2* is conditionally knocked out in RGCs at E13.5, neither glia nor OB interneuron generation is affected. Despite *Fezf2's* negligent influence on differentiation, our quit fraction and intermediate progenitor analysis of the *cKO* in the V/SVZ suggests that FEZF2 reduces the proliferative lifespan of RGCs and leads to preferential differentiation of RGCs into neural intermediate progenitors.

Acknowledgements

This study was funded by grants to B Chen (NIH R01 MH094589 and R01 NS089777). I would like to thank Bin Chen for being an inspiration through her tireless passion and enthusiasm for developmental neuroscience, for being an excellent and encouraging mentor and for never losing faith in me. Additionally, I would like to thank Jeremiah Tsyporin for being a best friend and the leader of our project. He brightened my day through our shared humor that always kept me going when I was losing faith. Simultaneously, his patience while teaching, guidance and unbelievable work ethic is an inspiration I will carry with me. None of this would be possible without David Tastad, who hired me as his undergraduate and taught me the skills required for research. Throughout the years his scientific expertise, advice and emotional support was essential to my success. I would like to thank Xiaoyi Guo for her positive attitude and helpful, friendly nature, Kendy Hoang for his comradery and Cosmo Hahn for his assistance with experiments. I would like to thank Mama for always knowing exactly the right thing to say to make me feel better, Papa for his insightful perspective and advice and my sister for being kind, funny and supportive. I would also like to thank my thesis committee, Professors Euiseok Kim and David Feldheim for their valuable feedback and knowledge. I would like to thank Professors Bill Saxton and Todd Lowe for being great teachers that continued to spark my curiosity and fascination of biology. I will never forget and am so grateful for the entirety of the Chen lab that has been a family to me during this unbelievably challenging, rewarding and enlightening time called Graduate school. I have never been so sure that I was exactly where I was supposed to be. The internal strength and perseverance this program cultivated in me has prepared me for my future.

Introduction

Striving to understand how consciousness and self awareness could arise from the signals sent between 100 billion neurons has been one of the greatest challenges of mankind (28). The brain develops through precise temporal and spatial generation of numerous types of neurons and the glia that support and regulate their function (31). Gene expression patterns dictate the characteristics that an immature postmitotic neuron will acquire. These include the type of neurotransmitter and receptor, the number of processes, cell body size, electrophysiological properties and their projections (32). The large surface area of the human cortex, with its dense neuron population and six layers folding and bending to form a complex labyrinth of sulci and gyri, is what gives humans the unique ability to perform higher cognitive tasks (32). Despite mice having smooth cortices, their cortex is also divided into six layers, analogous to the human cortex. Therefore, studying the development of their cortex provides humans insight into our own. Transcription factors, such as FEZ Family Zinc Finger 2 (*Fezf2*) which are conserved between mouse and human, are necessary for regulating the timely differentiation into specific cortical neuronal subtypes (16). Defects in cortical neurogenesis have been shown to lead to a variety of mental disorders and disabilities (36, 37). For example, Transcription factors such as FEZF2, TBR1, SOX5 and SATB2 have been linked to ASD and intellectual deficiency (29). Radial glial cells (RGCs) give rise to the cortical neurons and glia (38). Therefore, understanding the precise molecular mechanisms regulating RGC differentiation may prove critical for advancing our understanding of cognitive disorders and neurodegenerative diseases. This understanding will be invaluable for manufacturing new or improved therapeutics. This thesis strives to elucidate the perinatal role of *Fezf2* expression in the differentiation and proliferation of RGCs in the dorsal forebrain.

Anterior-Posterior and Lateral-Medial Embryonic Neural Development

The nervous system arises from a simple structure called the neural tube (31). The neural tube becomes specified along different axes based off of gradients of transcription factors and external signaling molecules. Starting at Embryonic day 7.5 (E7.5) in mice, the anterior-posterior axis of the developing nervous system is regulated by anterior transcription factor (TF) OTX2 and posterior TF GBX2 which determine the mid-hindbrain boundary (31). At E8.5, the neuroectoderm folds inward, forming the neural tube. Meanwhile, Fibroblast Growth factor 8 (Fgf8), which is critical for forebrain induction, begins being expressed at the anterior pole (31, 30). At E9.5 a singular forebrain vesicle separates into the telencephalic and diencephalic vesicles (6). The telencephalic vesicle develops into the telencephalon, which will develop into the cortex and other structures (6). By E10.5 and E11.5, opposing gradients of the transcription factors PAX6 and EMX2 specify regions of the cortex (31). High caudomedial expression of *Emx2* promotes development of the V1 region (7). Conservesly high expression of *Pax6* promotes development of the motor and somatosensory regions, which are the cortical regions examined in this study (31, 7).

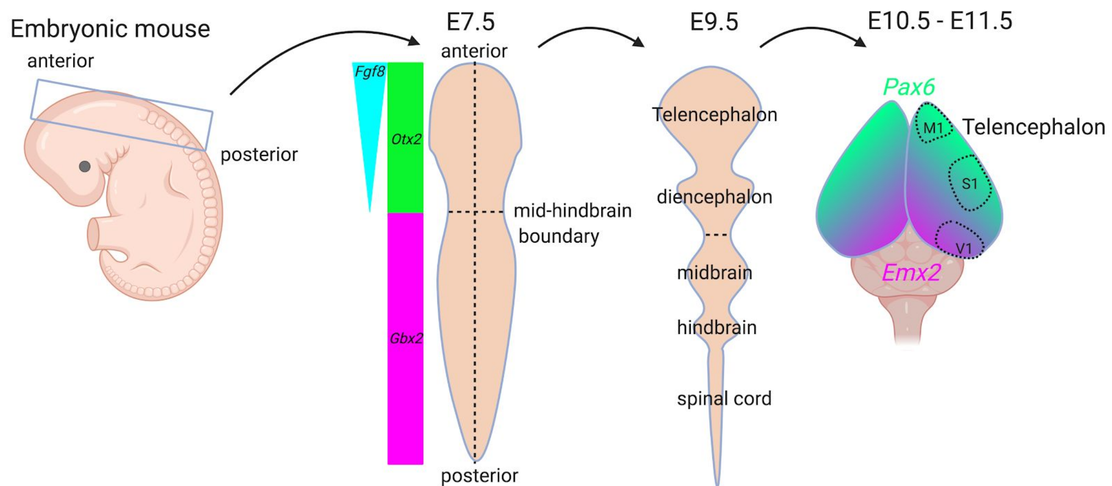


Illustration 1: Depiction of anterior-posterior neural development. Created on BioRender.

Dorsal-Ventral Embryonic Neural Development

Simultaneously, once the neural tube is formed, the dorsal-ventral axis is determined by the opposing concentration gradients of ventrally secreted Sonic Hedgehog (Shh), dorsally secreted Bone Morphogenetic Proteins (BMPs) and Wnt signaling protein (31, 39). The notochord, which is immediately ventral to the neural tube, secretes Shh which induces floorplate formation and a positive feedback loop in which more Shh is secreted from the floorplate (31). The floorplate is the most ventral part of the neural tube. The most ventral neural tissue with the highest Shh concentration triggers NKX2.2$^+$ and NKX6.1$^+$ cells. Cells with intermediate levels of Shh express *Olig2*, and tissue with little to no Shh exposure adopt a PAX7$^+$ dorsal fate (31). Neural stem cells (NSCs) in the dorsal forebrain and dorsally fated cell types are the focus of this study.

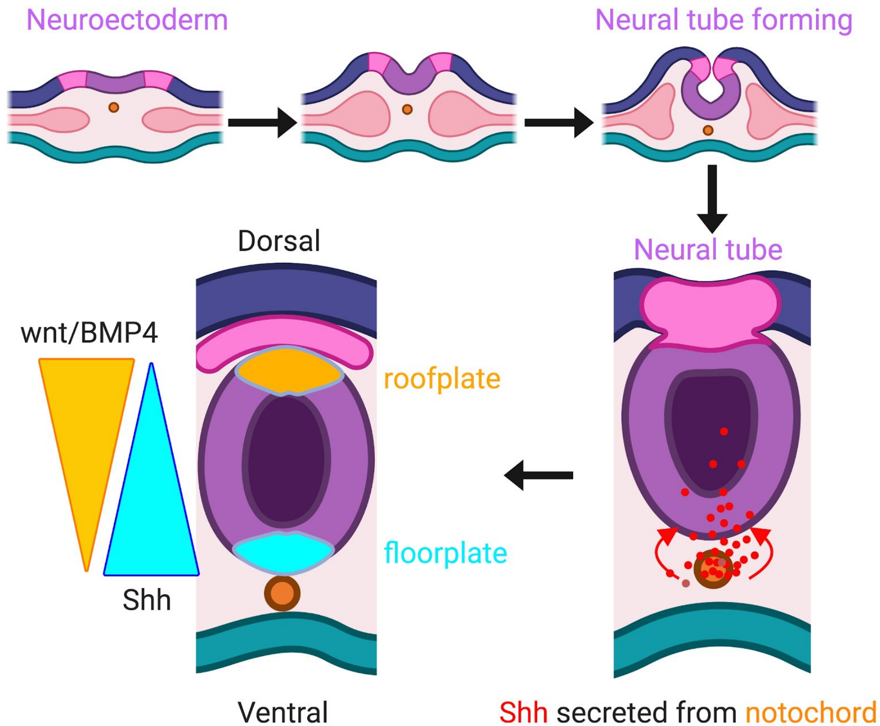


Illustration 2: Depiction of Dorsal-Ventral neural development. Created on BioRender.

Radial Glial Cells and the Development of the Neocortex

At E9, the telencephalon begins its development into the cortex (31). The internal neuroepithelium adjacent to the lateral ventricle is lined with apical progenitors (APs) (8). These APs have fibral extensions with endfeet that adhere to the basal pial membrane and exhibit interkinetic nuclear migration, such that the vertical movement of the nuclei is cell cycle dependent (9). During S and G2 phase, transport of the microtubule-associated protein Tpx2 from the nuclei to the apical process allows for apical nuclear migration along its own radial extension, so that mitosis occurs in the ventricular zone. Subsequently, the nucleus travels basally during G1 phase (9). APs initially undergo symmetrical division in order to increase the progenitor pool size (9). By E11, asymmetrical cell division begins in which the

progenitor daughter cell remains in the ventricular zone and the neuronal daughter cell inherits the radial fiber (9). Using the radial fiber, the postmitotic immature excitatory neuron travels radially which thickens the neuroepithelium as it gradually transforms into the cortex (10). Eventually, APs divide to generate neural intermediate progenitors cells (nIPCs) which reside in the subventricular zone where each nIPC will generate between 2-4 postmitotic excitatory neurons (10).

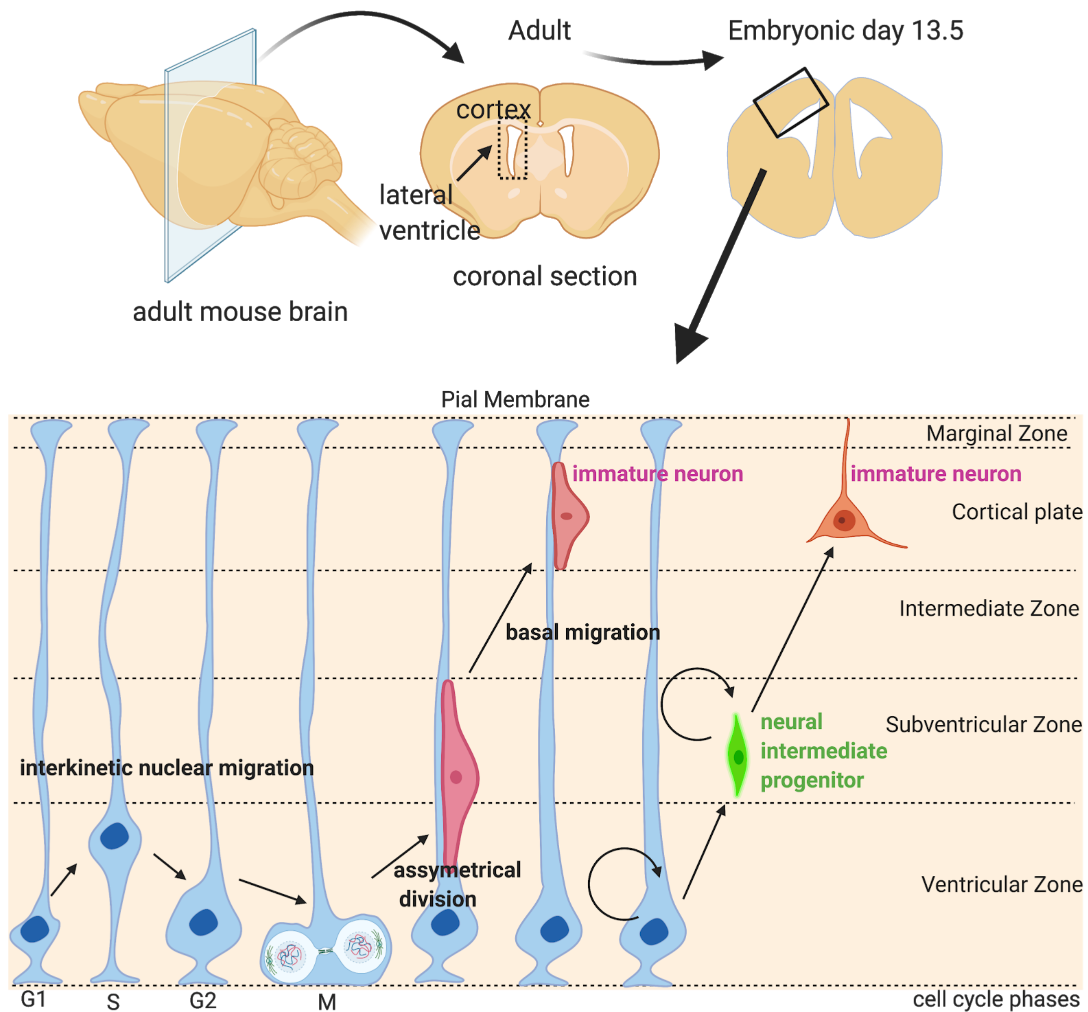


Illustration 3: Depiction of radial glial cell division and neuron generation. Newly born neurons migrate along radial glial extensions to the cortical plate. Created on BioRender.

The neocortex develops in an inside-out manner such that the neurons born first between E11 and E13 generate the deeper layers (V and VI), followed by the upper layers (II, III, IV) during E13 to E16 (31). Therefore, the upper layer neurons must travel past the deep layer neurons to reach their final destination (8, 9). Excitatory glutamatergic projection neurons constitute two-thirds of the neurons in the mammalian cerebral cortex and are critical for higher cognitive processing (40).

At E17, NSCs or RGCs switch from generating cortical neurons to generating olfactory bulb (OB) interneurons and cortical neural supporting glial cells: astrocytes and oligodendrocytes (8, 9). The remaining RGCs in the lateral ventricle continue to proliferate into adulthood, however at a much slower rate (41). FEZF2 is one of the transcription factors expressed in RGCs during cortical development and into adulthood, according to unpublished data from our lab.

FEZF2 in Cortical Development

Fezf2 was first discovered as an anterior neuroectoderm-specific gene in Xenopus and zebrafish (1). Phenotypes exhibited by Fezf2 mutant mice include abnormal craniofacial, gustatory, and respiratory morphology, increased mortality, premature aging and overall smaller body size (3). Mutants also display abnormal behaviors such as running in circles (3). FEZF2 is a transcription factor partially composed of an engrailed homology 1 (*Eh1*) repressor motif and six C2H2 zinc finger domains (42). THE EH1 domain interacts with TLE (Transducin-like enhancer of Split) transcriptional co-repressors to generate a strong transcription repression complex in xenopus (1). The zinc finger domains interact directly with DNA (12). Our lab came to this conclusion through generation of a *Fezf2-Bac-Enr; Fezf2*$^{-/-}$ mice which rescued the cortical phenotype of the *Fezf2* mutant mice (12). *Fezf2* is expressed in Layers 5 and 6 of the developing cortex in postmitotic excitatory neurons and is

essential for excitatory deep layer neuron specification in the mouse cortex (4). In the absence of *Fezf2* expression, axon fasciculation is prevented, most of these Layer V neurons no longer project to subcerebral areas such as the brainstem and spinal cord (4). As well as being required for proper axonal projection, it also determines dendritic morphology (4). A knockdown of *Fezf2* in Layer V of the primary motor cortex using silencing RNAs causes a reduction in cell body size, dendritic complexity, dendritic spine length and the radial length of basal dendrites and an alteration in the radial orientation of apical dendrites (4). Layer II/III neurons which usually project in a cortico-cortical manner were found to express Tbr1 (a deep layer marker) and instead project to subcerebral targets when Fezf2 was ectopically expressed (4).

Therefore, *Fezf2* expression is not only necessary for deep layer neuron fate specification, but also sufficient to induce characteristics specific to deep layer neurons when ectopically expressed (4). Markedly, Corticospinal motor neurons, which *Fezf2*'s expression is necessary to generate, are the same neurons which undergo apoptosis in the devastating neurodegenerative disease Amyotrophic lateral sclerosis (ALS) (5). This disease has a very rapid onset in which victims lose all motor abilities and eventually die from losing control of respiratory muscles (5). Understanding the molecular mechanisms underlying how specific cell types are initially generated may be key to determining how to combat their eventual degeneration.

FEZF2 in RGCs

Ectopic Fezf2 expression in upper cortical layers, late cortical progenitors and lateral ganglionic eminence progenitors is substantial to induce deep layer neuron characteristics, however not all *Fezf2* expressing RGCs (fRGCs) are lineage restricted to cortical excitatory deep-layer neurons (11, 16, 43, 44, 26, 45). Fezf2 is expressed in the dorsal and medial regions of the lateral ventricle from Embryonic day 10 into adulthood and only during a tiny fraction of this time are these neurons generated (11). Timed Lineage tracing using *Fezf2*

Cre-ER and a *RCEGFP* reporter showed that Fezf2$^+$ RGCs generate corticothalamic projection neurons (CThPN), subcerebral projection neurons (ScPN), callosal projection neurons (CPN), astrocytes and oligodendrocytes in a time dependent manner (11). Further research was conducted to determine at what stage of differentiation *Fezf2* expression became crucial for cortical development. At Postnatal day 7 (P7) the somatosensory cortex of *Fezf2* knockout mice (*KO*) and *Fezf2* conditional knockout mice, in which *Fezf2* was knocked out only in postmitotic neurons displayed the same cortical phenotype (12). Specifically, the cortex exhibited identical disruptions in the expression patterns of cortical deep layer specific transcription factors, such as BHLHB5, CTIP2 and SOX5 when *Fezf2* was completely absent as when it is only absent in postmitotic neurons (12). This result indicates that deep layer specification is decided within the immature FEZF2$^+$ postmitotic neuron and not at the level of its progenitor cell (12). Further, if neuronal cell fate specification occurred only within the RGC, then knocking out *Fezf2* in the postmitotic neuron would not influence neuronal cortical development.

FEZF2 in RGCs does not play a fundamental role in regulating cortical neurogenesis. Nevertheless, *Fezf2* is expressed in the cortical RGCs during development and into adulthood. This thesis strives to understand how *Fezf2* expression in RGCs impacts their proliferation and differentiation.

Our Technique

To address how *Fezf2* expression in RGCs influences differentiation, Jeremiah Tsyporin and I performed timed lineage tracing of Fezf2$^+$ RGCs from E14.5 to P7 in *Fezf2* knockout and wildtype mice. I quantified the percentage of GFP$^+$ cells that express oligodendrocyte and astrocyte specific protein markers in the cortex. I analyzed the same markers in the cortex, as well as OB interneuron specific markers in the *Fezf2* RGC

conditional knockout (*cKO*), *Fezf2*$^{fl/-}$ *hGFAPcre*$^{+/-}$. To address proliferation, we analyzed the quit fraction and the ratio of dividing nIPCs in the VZ and SVZ of the *cKO* at E17.5 and P0.

Results

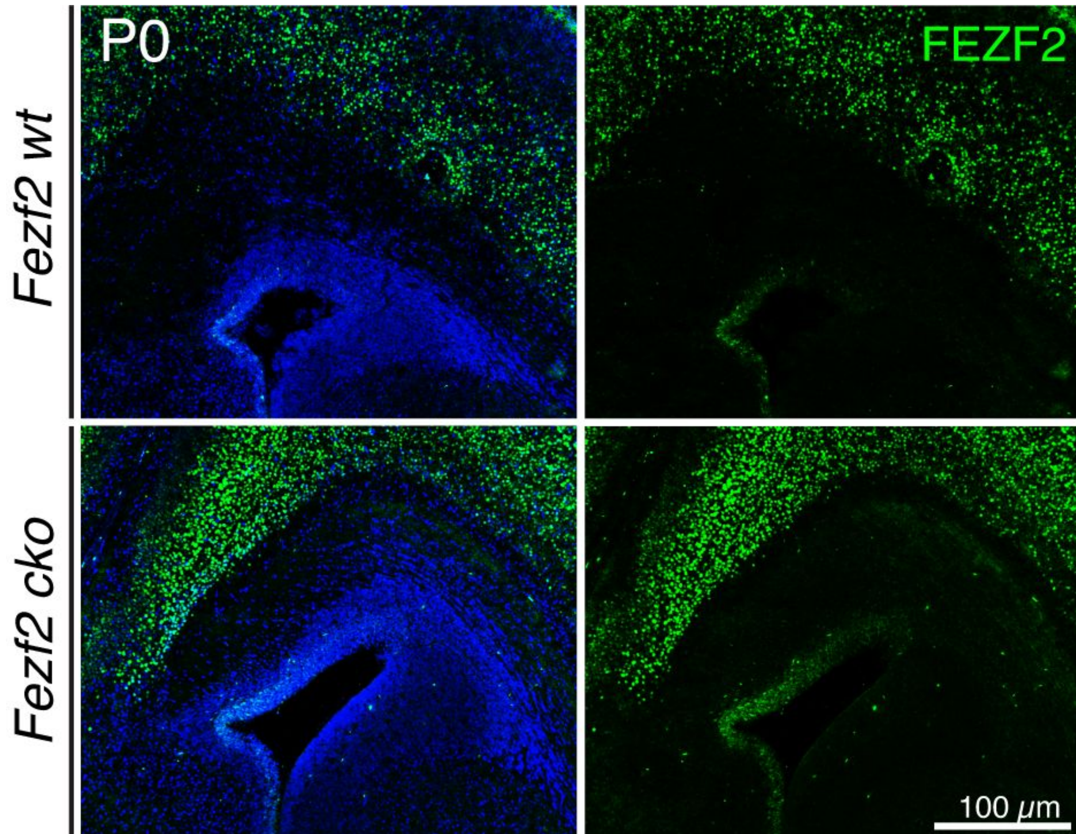
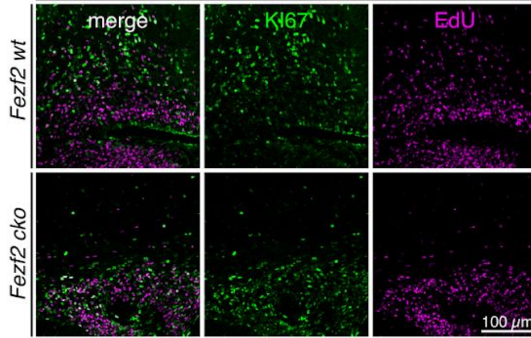
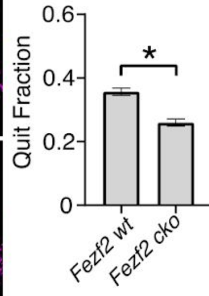


Figure 1: FEZF2 immunostaining in the dorsal VZ at P0.

Figure 2

A. EdU E18.5, analysis P0





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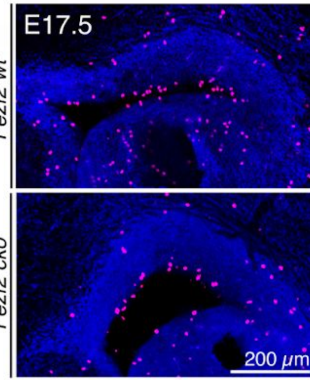
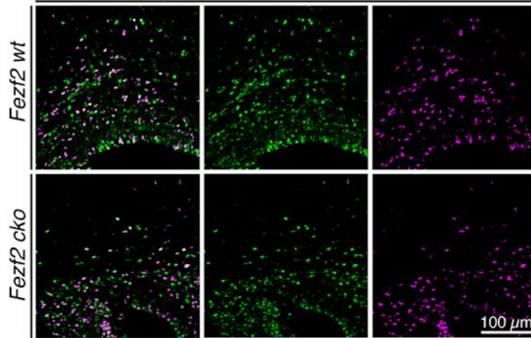
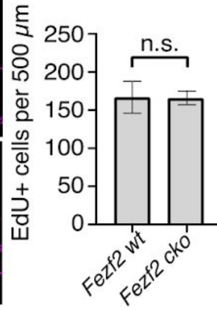
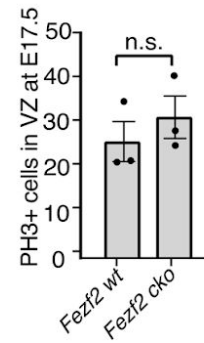


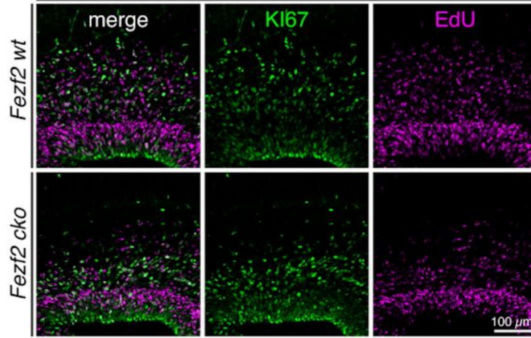
B. EdU P0, analysis +2 hours

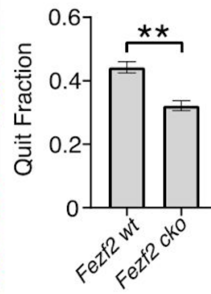




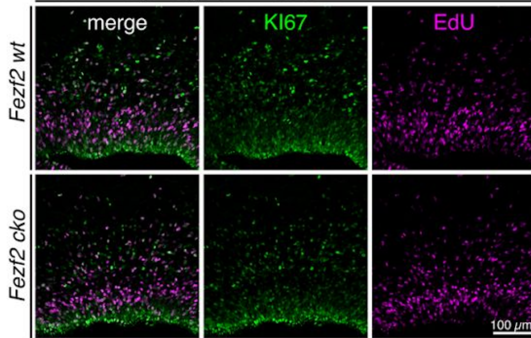


C. EdU E16.5, analysis E17.5





D. EdU E17.5, analysis +2 hours



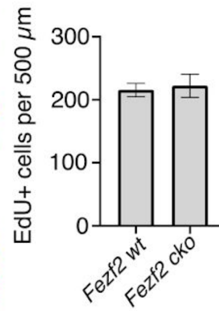
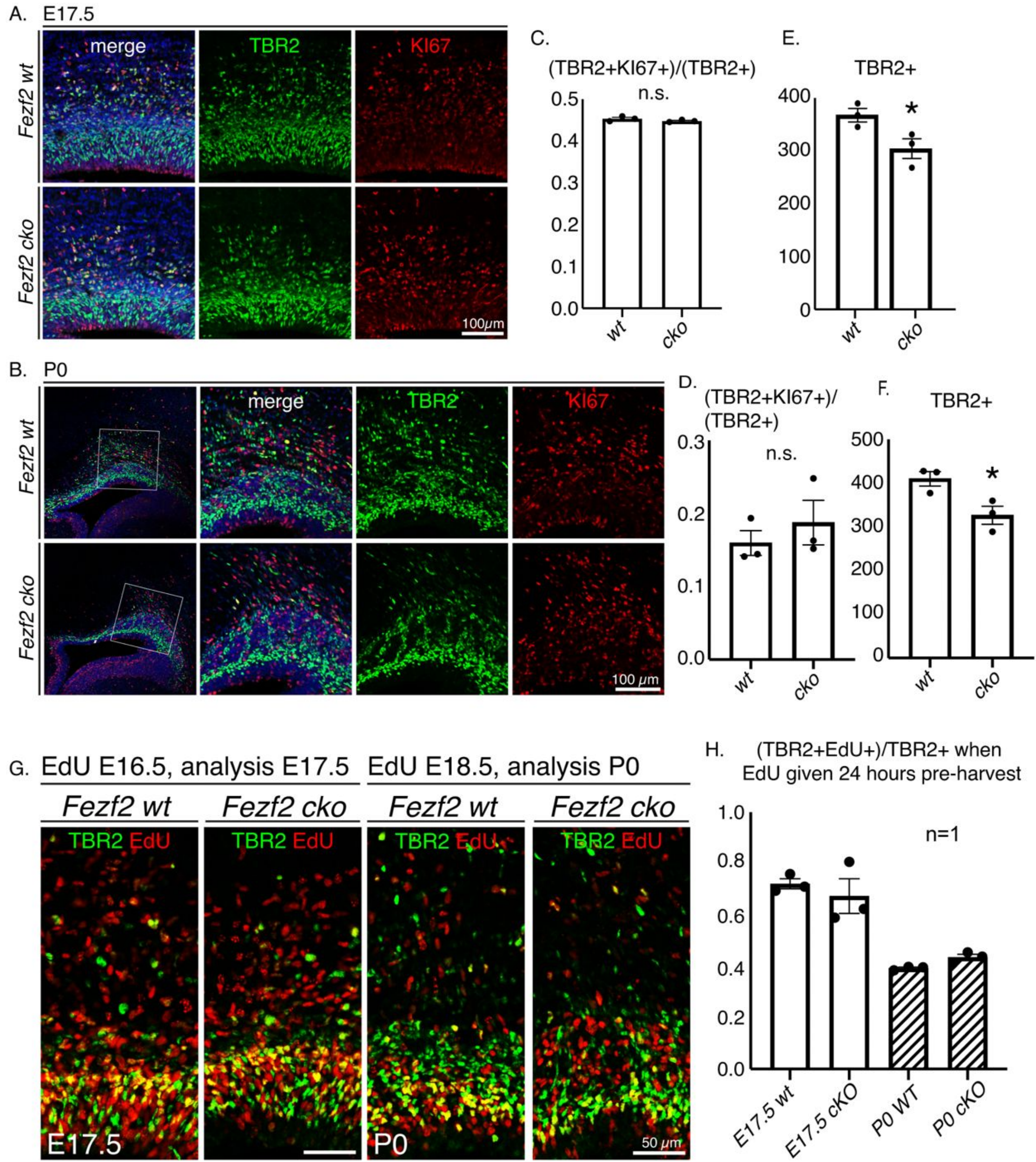


Figure 2: Absence of FEZF2 in RGCs lengthens the proliferation period of NSCs in the VZ at E17.5 and P0. A-E. EdU, KI67 and PH3 labeled cells in the dLV. **A-D** shows KI67^{+} cells clustering closer to the ventricular zone in the *cKO*(bottom panel) than in the control(top panel.) There is a decrease in quit fraction in the *cKO* when EdU is administered at E18.5 and brains collected 24 hours later at P0 **(A)** and when EdU is administered at E16.5 and brains collected 24 hours later at E17.5**(C)**. **B.** There is no significant change in the number of proliferating cells when EdU is given 2 hours before harvesting at P0**.** (n.s. = not significant) **A-C.** n=3 brains, 3 sections per brain (*p<0.05, **p<0.01, student's paired T-test). **D.** Significance cannot be determined. n=1 brain, 3 sections per brain. **E.** There is no significant change in the total number of cells undergoing mitosis at E17.5. n=3 brains, 3 sections per brain (*p<0.05, **p<0.01, student's unpaired T-test). Error bars represent SEM.

Figure 3.



I.

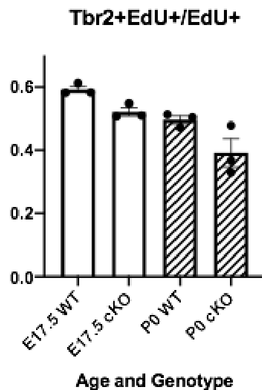


Figure 3: Absence of FEZF2 in RGCs results in decrease of Intermediate progenitors perinatally. A-B. Immunohistochemistry of KI67$^+$ and TBR2$^+$ cells in the dLV of controls(top panel) and *cKO*(bottom panel) at E17.5 and P0. **C-D.** There is no significant change in the rate of IP proliferation, quantified by (TBR2$^+$KI67$^+$)/TBR2$^+$. **C-F.** Each dot represents the average of three sections from the same brain. n=3 brains, 3 sections per brain (student's unpaired T-test). Error bars represent SEM. **E-F.** There is a significant decrease in the number of TBR2$^+$ cells at E17.5 (p= 0.0337) and P0 (p= 0.0494) in the *cKO*. (Student's unpaired t-test, *p<0.05). **G**. The Ventricular and subventricular zone of the dLV in the *cKO* and *wt* illustrate a similar phenotype at E17.5 and slight decrease of TBR2$^+$ IPs at P0. **H.** The ratio of TBR2$^+$ IPs that are in the process of dividing between E16.5 and R17.5 and between E18.5 and P0. **I.** The ratio of dividing cells that differentiate into TBR2+ IPs within a 24 hour window. **H-I.**The white and striped bars represent littermate controls. Each dot represents the quantification from one section. Significance cannot be determined. n=1, 3 sections per brain. Error bars represent SEM.

Absence of Fezf2 increases RGC proliferation but reduces Intermediate Progenitor differentiation

Antibody detection shows that *Fezf2* is expressed in the cortical VZ at P0 (Figure 1). In order to investigate how FEZF2 affects RGC proliferation and differentiation into TBR2$^+$ nIPCs, *Fezf2* was conditionally knocked out in RGCs using *Fezf2$^{fl/-}$ hGFAP-Cre* mice(*cKO*). To determine if Fezf2 affects cell cycle exit, I administered EdU to E16.5 pregnant mice, and collected the brains 24 hours later. Then the quit fraction was determined by the ratio of EdU$^+$ cells that are KI67$^-$. KI67 labels all cells in active phases of the cell cycle (G1, S, G2, M) (33). A significantly reduced quit fraction in the VZ/SVZ at E17.5 and P0 in the *cKO* indicates the

NSCs are exiting the cell cycle at a lower rate, and thereby exhibiting a lower rate of differentiation (Figure 2A, C).

By calculating the fraction of TBR2$^+$ cells that are KI67$^+$ at E17.5 and P0, I found that IP proliferation is unaffected by FEZF2 (Figure 3C, D). TBR2 is present in nIPCs and in the early postmitotic neurons they give rise to (34). To further verify this result, I determined the fraction of TBR2$^+$ cells that are still dividing between E16.5 and E17.5 and between E18.5 and P0, by giving EdU 24 hours prior to harvesting and EdU$^+$TBR2$^+$/EdU$^+$ appears to remain unchanged based on the data presented (Figure 3G, H). These results suggest that FEZF2 does not influence the rate of divisions of IPs. However, there is a 17% and 20% decrease in the total number of TBR2$^+$ cells in the *cKO* at E17.5 and P0, respectively (Figure 3E, F). The total number of dividing cells (EdU$^+$ and PH3$^+$) at E17.5 and at P0 remains unchanged, which indicates that the *cKO* reduction in TBR2$^+$ cells is not caused by a shortage of RGCs. However, KI67$^+$ and EdU$^+$ cells are clustering closer to VZ in the *cKO*, which suggests a lack of RGC differentiation (Figure 2). It also appears that the total number of dividing cells differentiating into TBR2$^+$ IPs is decreased in the cKO (Figure 3I). These data suggest that the *cKO* reduction in TBR2$^+$ cells is not caused by a change in the rate of IP proliferation, but rather a decrease in the rate at which RGCs differentiate into IPs. Therefore, *Fezf2* expression in RGCs ensures their differentiation into TBR2$^+$ IPs. In conclusion, *Fezf2* maintains the balance between RGC self renewal and neuronal differentiation.

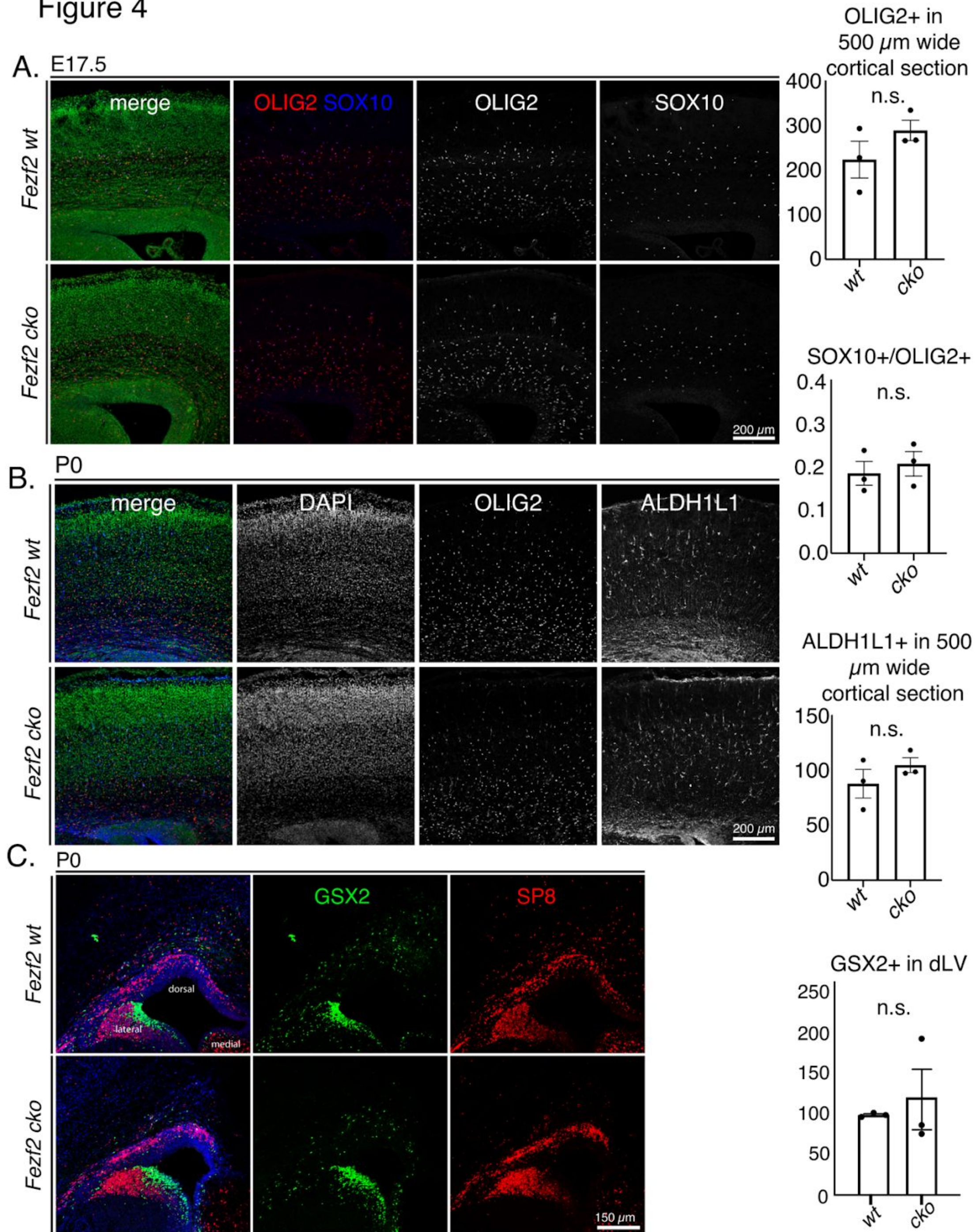


Figure 4: FEZF2 does not influence the cortical density of Glial subtypes perinatally. **A-B.** Quantifications and images taken from the M1 region of cortical plate. **A.** At E17.5, there is no significant change in the total number of glial cells (OLIG2^{+}) or the ratio of SOX10^{+} oligodendrocytes compared to the total number of glial cells between the control (top panel)

and Fezf2 *cKO* (bottom panel). **B.** At P0, there is no significant change in the number of astrocytes (ALDH1L1$^+$). **C.** At P0, there is no significant change in the number of GABAergic OB interneuron progenitors (GSX2$^+$) in the VZ and SVZ. GSX2$^+$ cells give rise to SP8$^+$ OB interneurons. **A-C.** Data gathered from n=3 brains, 3 sections per brain (student's unpaired t-test, n.s.=not significant). Each dot represents the average of three sections from the same brain. Error bars represent SEM.

Absence of FEZF2 in RGCs likely does not impact glial or OB interneuron generation

In order to investigate how FEZF2 affects RGC differentiation, *Fezf2* was conditionally knocked out in RGCs using the same *cKO*. *hGFAP-Cre* begins being expressed at E13.5, at which time point RGCs have already given rise to deep layer neurons, but most upper layer neurons, glia and OB interneurons have yet to be generated (11, 47). GSX2$^+$ progenitors differentiate into SP8$^+$ cells, which was used to mark immature and mature GABAergic OB interneurons (Figure 4C). At P0, there is no significant difference in the number of GSX2$^+$ cells in the dorsal VZ and SVZ (Figure 4C). Nor is there a significant change in the density of cells expressing the astrocyte marker ALDH1L1 in the cortex (Figure 4B). These results indicate that FEZF2 is not necessary for regulating OB interneuron or astrocyte generation between E13.5 and P0. Similarly, at E17.5, there is no significant difference in the density of cortical OLIG2$^+$ cells, which is expressed in most glial cell types (Figure 4). Additionally, the ratio of SOX10$^+$ expressing oligodendrocytes relative to the total of Olig2$^+$ glial cells is not significantly changed between the *cKO* and control (Figure 4A). Hence, *Fezf2* expression in RGCs is not necessary for regulating oligodendrocyte generation.

Even though there was not a significant change in the total number of cells expressing these markers, there does appear to be a slight increase in OLIG2$^+$ and ALDH1L1$^+$ cells in the *cKO*. If there is a slight increase that would be found to be significant with analysis of more brains, then this result would be coherent with the data derived from analysis of TBR2$^+$ cells. The *cKO* showed a reduction in TBR2$^+$ IPs, which are neuronal

progenitors, so it would make sense that the RGCs would instead preferentially differentiate into oligodendrocytes or astrocytes.

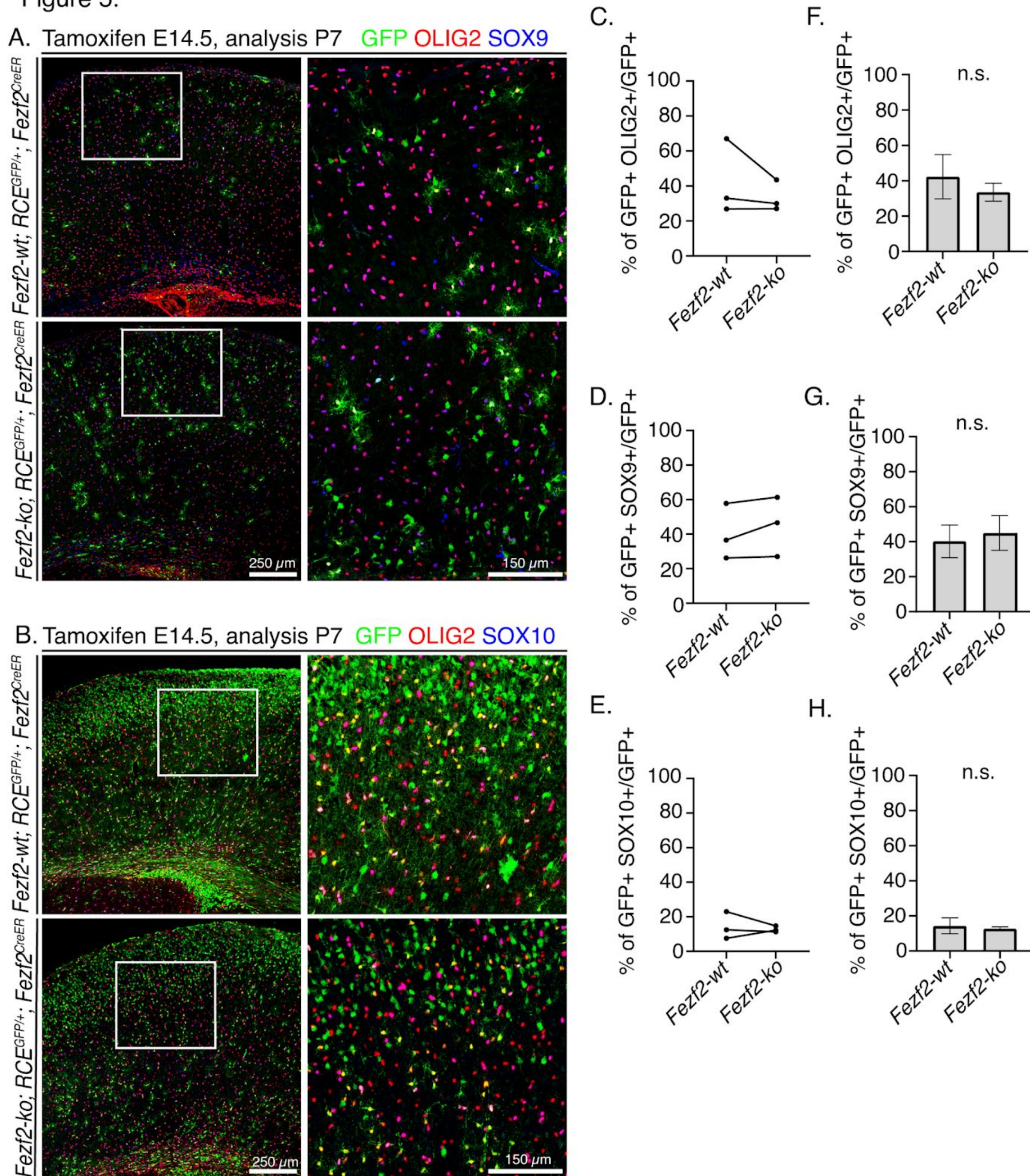


Figure 5: Fezf2 expression in RGCs does not influence post-mitotic differentiation potential. A-B. Temporal lineage tracing was performed by giving Tamoxifen at E14.5 and harvesting Fezf2$^{-/-}$ Fezf2CreER$^{+/-}$ RCE-GFP$^{+/-}$ and Fezf2$^{+/+}$ Fezf2CreER$^{+/-}$ RCE-GFP$^{+/-}$ brains at P7. **A.** OLIG2 labels all glial subtypes and SOX9 is specific to astrocytes as can be seen by starlike morphology of triple labeled cells. **B.** SOX10 labels a subtype of oligodendrocytes. **C-E.** The proportion of GFP^{+} cells that express either OLIG2, SOX10 or SOX9 in 1.6 mm^2 regions of the motor cortex. Each dot represents the average of 3 cortical sections from one brain and the line is drawn between littermate controls. A littermate controlled student's

paired t-test was performed for the lineage tracing since it is expected that the ratios of GFP$^+$ clones of a specific glial subtypes may vary slightly between litters. This is because the time of tamoxifen injection relative to the time point of embryonic development is dependent on the time at which the mouse was impregnated. **F-H.** The mean of the data in C-E, respectively. There was no significant difference for any of the markers. n=3 brains, 3 sections per brain. (Student's paired T-test). Error bars represent SEM.

Lineage Tracing suggests knocking out Fezf2 **does not impact identity of RGC clones**

Unlike analysis of the *Fezf2 cKO*, lineage tracing allows for determining the cell autonomous function of FEZF2 in RGC differentiation by analysis of the RGC clones. Using this method prevents a potential compensatory mechanism from skewing the data on how FEZF2 influences differentiation. A tamoxifen inducible *Fezf2-CreER*^{T2} allele was used to perform lineage tracing in Fezf2$^{+/+}$ and Fezf2$^{-/-}$ mice. Using *Fezf2-CreER*^{T2}, Cre is expressed in the same RGCs that express FEZF2 and RCEGFP is a reporter that allows for visualization of clones of fRGCs (11). Previously, it was shown that at E16.5 RGCs begin to transition from generating neurons to glial cells (11). We administered tamoxifen at E14.5 to label a substantial portion of all clonal subtypes generated during cortical development. Specifically, between E14.5 and P21, 59% of RGCs clones are neurons, 23% are astrocytes and 15% are oligodendrocytes (11).

Since the *cKO* exhibits a reduced quit fraction and a decrease in nIPC differentiation, one possibility is that *KO* RGC clones would alternatively generate a higher proportion of glia and OB interneurons. However, there was no significant difference between the *Fezf2 KO* and *WT* in the percentage of Sox10$^+$, Sox9$^+$ or Olig2$^+$ GFP$^+$ colabeled cells out of the total of GFP$^+$ cells (Figure 5). The averages for the *KO* and *WT* were: SOX9$^+$(45% vs. 40%), SOX10$^+$(14% vs. 13%) and OLIG2$^+$(34% vs. 42%), respectively. These data indicate Fezf2 expression in RGCs likely does not impact lineage differentiation into oligodendrocytes, astrocytes or neurons.

Discussion

Using a Fezf2 antibody, we showed that *Fezf2* is expressed in the cortical RGCs (Figure 1, 35). Immunohistochemistry shows a decrease of FEZF2 Antibody in the VZ of the *cKO* (Figure 1). In the control, the stain shows the presence of functional FEZF2. The loxP sites in the *Fezf2$^{fl/fl}$* construct flank exon 2 of *Fezf2*, such that when Cre is expressed, exon 2 is excised from the genome (12). Hence, in the *cKO*, FEZF2 signal can be visualized because the truncated nonfunctional protein expressed in hGFAP-Cre^{+} progenitors may be binding to the FEZF2 antibody. An *in situ* experiment with a probe specific to the excised exon would be a more accurate depiction of FEZF2 expression and very little to no signal would be expected in the VZ/SVZ of the *cKO*.

The quit fraction results indicate that FEZF2's function in RGCs is to promote cell cycle exit. The same conclusion was drawn from electroporation of *Fezf2* overexpression and EGFP reporter plasmids into human cortical progenitor cells *in vitro* (20). This experiment showed that *Fezf2* overexpression resulted in a decreased proportion of KI67^{+} cells (20). Similarly, FEZF2 was found to be required for maintaining quiescence in adult zebrafish dorsal telencephalic (DTel) radial glia-like progenitors (RGLs) (23). *Fezf2* expression was found to be high in quiescent and low in proliferative DTel RGLs and in postnatal mouse hippocampal NSCs (23). Gene expression analysis on FACS purified cells showed that FEZF2 was an upstream regulator of many notch pathway genes, particularly *her4.1*, which may be critical for FEZF2's regulation of the cell cycle in adult vertebrate neurogenesis (23).

FEZF2 is not only involved in reducing proliferation of stem cells, but also has the same function in multiple cancers by acting as a tumor suppressor. For example, FEZF2 is a novel biomarker in low‑differentiated Colorectal carcinoma tissues and mRNA microarray data showed that *Fezf2* mRNA expression was dramatically decreased in bladder cancer tissues when compared to adjacent non-tumor tissue (22, 21). To test FEZF2's function, the authors ectopically expressed *Fezf2* in multiple bladder cancer cell lines and saw a drastic

decrease in proliferation. Even more notably, overexpression of *Fezf2* eradicated tumorigenicity of bladder cancer *in vivo*. The authors suggest FEZF2 regulates the aggressiveness of the cancer primarily via the NF-κB signaling pathway (21). Consistent with the findings shown here, FEZF2 appears to have the widespread function in vertebrates of preventing or halting cell division during embryonic development and into adulthood. The NF-κB pathway has been found to be integral in human cancer for FEZF2's regulation, while in NSCs of zebrafish, it was the notch pathway. However, it has also been found that there is crosstalk between these two pathways, so more research would need to be done to discover how similar the mechanism of action is of FEZF2 across vertebrate species, cell type and age (24).

So far FEZF2's ability to delay or prevent proliferation is fairly well understood and unanimously agreed upon. However, FEZF2's role in differentiation is more complex and much more dependent on cellular subtype. For example, in the retina, FEZF2 is required for differentiation of cone OFF bipolar cells, while in the cortex it is necessary and sufficient for deep layer neuron projection and morphology (25,12). When *Fezf2* is ectopically expressed in neural striatal progenitors and upper layer neurons it reprograms them to generate clones that express protein markers and exhibit morphology associated with glutamatergic corticospinal neurons (26, 45).

Our data from the *cKO* and lineage tracing *KO* indicate little to no change in glial or OB interneuron generation (Figure 4, 5). The density of cells expressing oligodendrocyte, astrocyte and OB interneuron protein markers is only slightly altered in the *cKO* and the lineage tracing indicates no change in the cell fate of GFP^{+} cells either (Figure 4, 5). There are many more potentially useful experiments that may explain why the proliferation analysis is at odds with the lineage tracing results. For example, the glial cell markers at E17.5, P0 and P7 were only counted in the cortex, not the SVZ or white matter. It is possible that more glial cells are present in the *cKO*, but their migration could be delayed. If there is no change at P0 or P7 in the number of glial cells in the white matter or SVZ, then perhaps even though

the quit fraction indicates RGCs proliferate for longer in the *cKO*, maybe a lack of FEZF2 causes premature differentiation into glia and a higher rate of immature glia undergoing cell death. This could be determined by performing a caspase stain analyzing Caspase^{+}OLIG2^{+}/OLIG2^{+} (or another cell type specific marker).

Additionally, the lineage tracing performed in our experiment identified the clones of RGCs that would have expressed *Fezf2* in the *Fezf2 KO* (Figure 5). The data from this genotype could have been compromising since Fezf2 was knocked out in the whole animal. *Fezf2* is normally expressed during neural tube development and the developing brain may have already compensated for a lack of *Fezf2* expression. In order to more accurately assess FEZF2's function in a clinically relevant way, our lab is electroporating Cre into the lateral ventricle of *Fezf2*$^{fl/pl}$ *RCEGFP*$^{+/-}$ and *Fezf2*$^{fl/wt}$ *RCEGFP*$^{+/-}$ mice at E14.5 and assessing the identities of the GFP^{+} clones at P7. This experiment allows for normal development so that any potential external signaling to compensate for a lack of FEZF2 during the whole of development is avoided. Therefore, the focus is only on the fate of a few NSCs in which *Fezf2* is knocked out.

Despite some discrepancies in our data, a need to investigate further using other methods and also a closer examination of the phenotypes of the aforementioned genotypes, our *cKO* and lineage tracing data so far shows that knocking out *Fezf2* completely or in RGCs at E13.5 has either no or minimal effect on the generation of glial subtypes or OB interneurons. In other words, *Fezf2* expression in RGCs, at least after E14.5 does not dictate post mitotic differentiation potential. This data suggests that levels of *Fezf2* expression in RGCs *in vivo* does not alter the ultimate cell identity of RGC clones. An absence of *Fezf2* in RGCs may not influence cell fate but ectopic overexpression of RGCs does.

Overexpression of *Fezf2* at P4 using lentiviral injections in the dorsal VZ found that ectopic expression of *Fezf2* reprograms SVZ NSCs to become pyramidal-like glutamatergic neurons instead of GABAergic granule cells (27). However, *Fezf2* overexpression did not

alter the identity of transit-amplifying progenitors or neuroblasts that were bound for OB interneuron fate (27).

FEZF2's fairly well understood function in regulating the cell cycle in a variety of cell types may be important for designing novel cancer treatments. Further understanding transcriptional regulators, such as FEZF2 and their function in differentiation *in vivo* and their potential for reprogramming multipotent progenitors postnatally is of critical importance for treating neurological diseases and disorders that are currently incurable, such as autism, schizophrenia, ALS and other neurodegenerative diseases.

Methods

Animals

Experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at University of California at Santa Cruz. The morning of vaginal plug detection and the day of birth was termed E0.5 and P0, respectively. Analysis was performed irrespective of sex.

hGFAP-Cre (JAX no. 004600), *RCE-GFP, Fezf2$^{-/-}$, Fezf2$^{fl/fl}$* mice were described previously (15, 13, 16, 17). The driver mouse line *Fezf2-CreER^{T2}* was generated via alteration of the *Fezf2 BACRP23-141E17* (14).

The *cKO* is *Fezf2$^{fl/-}$ hGFAP-Cre* and the control is *Fezf2$^{fl/+}$*. The knockout *Fezf2$^{-/-}$ Fezf2-CreER^{T2} RCE-GFP* and control *Fezf2$^{+/+}$ Fezf2-CreER^{T2} RCE-GFP* were analyzed for lineage tracing. PCR used to determine genotypes (Table 2).

Immunohistochemistry

Trans-cardiac perfusion was used to circulate 1X PBS and 4% paraformaldehyde in 1X PBS. Post-fixation of brains was achieved by submerging in 4% paraformaldehyde and 0.1% saponin in 1X PBS overnight at 4°C, followed by cryoprotection in 30% sucrose in PBS. Immunohistochemistry was conducted according to standard protocols. 25-μm-thick brain sections were washed with 0.03% Triton X-100 in 1XPBS for 15 minutes. Slides were submerged in citrate buffer (10mM citric acid monohydrate, 0.05% Tween-20, pH 6.0), boiled 5 times in a microwave and then rested in the solution until they cooled to RT. This was followed by a 30 minute blocking period with 5% horse serum, 0.03% Triton X-100 in 1XPBS. Upon removal of the blocking buffer, primary antibodies (diluted in the blocking buffer) were applied to the slides for 24 hours at 4°C (see table 1). The slides were then washed with 1XPBS, followed by a 2 hours RT incubation of secondary antibodies conjugated to Alexa 488, Alexa 546, or Alexa 647 derived from Jackson ImmunoResearch and Invitrogen. The

slides were washed with 0.03% Triton X-100 in 1XPBS for 25 minutes and then stained for DAPI for 20 minutes prior to being mounted using Fluoromount-G™.

EdU labeling and Quit Fraction

Timed pregnant *hGFAPcre*$^{+/-}$ *Fezf2*$^{+/-}$ bred to *Fezf2*$^{fl/fl}$ mice were injected with a single dose of EdU (25mg/kg; Thermo Fisher Scientific, E10187) at E16.5 and brains were collected at E17.5 exactly 24 hours later or EdU was given at E18.5 and brains were collected 24 hours later at P0. Quit fraction was determined by KI67^{-}EdU^{+}/EdU^{+} cells in the VZ and SVZ. For 2 hour analysis, the procedure was the same except that at E17.5 the pregnant mouse was injected prior to retrieving pups and at P0 the pups were injected.

EdU visualization was performed after the washing process of primary antibodies was complete by incubating slides for 45 minutes in a click-chemistry reaction containing the following reagents per 1 ml of reaction: 950ul 100mM Tris PH 7.4, 40ul 100 mM CuSO4, 10ul 200 mg/mL sodium ascorbate, and 1ul azide 488 or 555. *cKO (Fezf2*$^{fl/-}$ *hGFAPcre*$^{+/-}$) and control (*Fezf2*$^{fl/+}$) littermates were analyzed.

Lineage Tracing

Timed pregnant *Fezf2*$^{+/-}$ *Fezf2-CreER*^{T2} bred with *Fezf2*$^{+/-}$*RCE-GFP*$^{+/-}$ were administered Tamoxifen orally at 150 mg/kg at E14.5 and brains were collected at P7. Knockout (*Fezf2*$^{-/-}$ *Fezf2-CreER*^{T2} *RCEGFP*$^{+/-}$) and wildtype (*Fezf2*$^{+/+}$ *Fezf2-CreER*^{T2} *RCEGFP*$^{+/-}$) were analyzed.

Image acquisition and quantification

The Zeiss 880 confocal microscope was used to attain images for quantitative analyses. Laser power and gain were fine-tuned so that only a few cells were saturated. Photoshop was used to hand count single z-slices. For *cKO* analysis, 500 μm wide cortical regions were counted. KI67^{+}, TBR2^{+}, EdU^{+} cells were counted in 250 μm wide regions and for PH3^{+} and

GSX2$^+$ cells the whole dLV was counted. For lineage tracing analysis, 1.6 mm^2 regions of all 6 cortical layers were counted. For each brain and marker, analysis was performed on 3 to 4 sections derived from the S1 or M1 regions. The anterior-posterior, medial-lateral positions of the quantified regions for the genotypes of comparison were matched as closely as possible. The number of particles were counted using FIJI and each particle is considered a cell.
Only single Z-slice confocal images were used in cell quantifications. For each genotype and each age, between 1 and 3 different brains were analyzed. Data are shown as mean ± SEM . Statistical analysis was performed using GraphPad Prism 5.0. Statistical significance was only determined for experiments that had n=3. T-tests assuming gaussian distribution were used to determine significance. Significance was set as * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$ and **** for $p<0.0001$ all significance tests.

A paired t-test was performed for all lineage tracing and EdU data in which only counts within the same litter were compared since Edu is injected and may vary slightly between mice. Unpaired t-test were used for the remaining data.

Table 1. The following antibodies were used in this study. *M=Monoclonal; P=Polyclonal

Antibody	Host (M or P)	Cat. Number	Dilution	Immunogen	Source
ALDH1L1	Rabbit (P)	ab103935	1:1000	Synthetic peptide within Mouse ALDH1L1 aa 300-400 conjugated to keyhole limpet haemocyanin.	abcam
GFP	Chicken (P)	GFP-1020	1:500	Recombinant GFP protein emulsified in Freund's adjuvant	Aves Labs
GSH2	Rabbit (P)	ABN162	1:1000	KLH-conjugated linear peptide corresponding to the C-terminus of mouse Gsh2	EMD Millipore
OLIG2	Mouse (M)	MABN50	1:500	Recombinant protein corresponding to human Olig2	EMD Millipore
PHH3	Mouse (M)	9706	1:250	KLH-conjugated Synthetic peptide to SER10 phosphorylated Histone 3	Cell Signaling Technology
TBR2	Rabbit(P)	ab23345	1:500	Synthetic peptide conjuaged to KLH derived from within residues 650 to the C-terminus of MouseTBR2/Eomes	abcam
SOX10	Goat (P)	AF2864	1:200	E. coli-derived recombinant human SOX10	Bio-techne
SP8	Goat (P)	sc-104661	1:2000	Peptide mapping to C-terminus of human Sp8	Santa Cruz Biotechnology
KI67	Mouse (M)	550609	1:50	Human KI-67	BD Pharmingen
SOX9	Rabbit		1:1000	Recombinant fragment within Human SOX9 aa 150-300 (internal sequence).	Abcam

Table 2: Primers used for Genotyping.

Gene target	BC #	Primer sequence
RCE-GFP	BC 1553	CCC AAA GTC GCT CTG AGT TG
	BC 1554	GAA GGA GCG GGA GAA ATG GAT
	BC 1555	CCA GGC GGG CCA TTT ACC GTA
Generic-Cre	BC 123	ACC AGA GAC GGA AAT CCA TCG CTC
	BC124	TGC CAC GAC CAA GTG ACA GCA ATG
Fezf2-PLAP	BC 164	CAC CCC GGT GAA CAG CTC CTC GCC CTT GCT CAC CAT
	BC 202	CTG CAT GGC TCG GAA CGC ATC TCC TTG GCG GTG GGG GAA AGA G
Fezf2-WT	BC 252	TTG AAT GCA AAT GGG TGA CCG GGC CG
	BC 253	GTT TTA GAA GTG GCC GGT GAC GCT CC
Fezf2-flox	BC 1563	CTG CCT TGT ACA CCTTTC TCT
	BC 1564	GAG ACC TAG GCA AGG GAC AGT
Fezf2-CreER	BC 1551	TCA CGC GGA GGG GAA GAT GTT
	BC 1552	GTG GCA GCC CGG ACC GAC GAT

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