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Understanding the role of fezf2 in adult neural stem cell maintenance and fate: A study in zebrafish

Author
Berberoglu, Michael A.

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Understanding the role of fezf2 in adult neural stem cell maintenance and fate: A study in zebrafish

by

Michael A. Berberoglu

DISSERTATION

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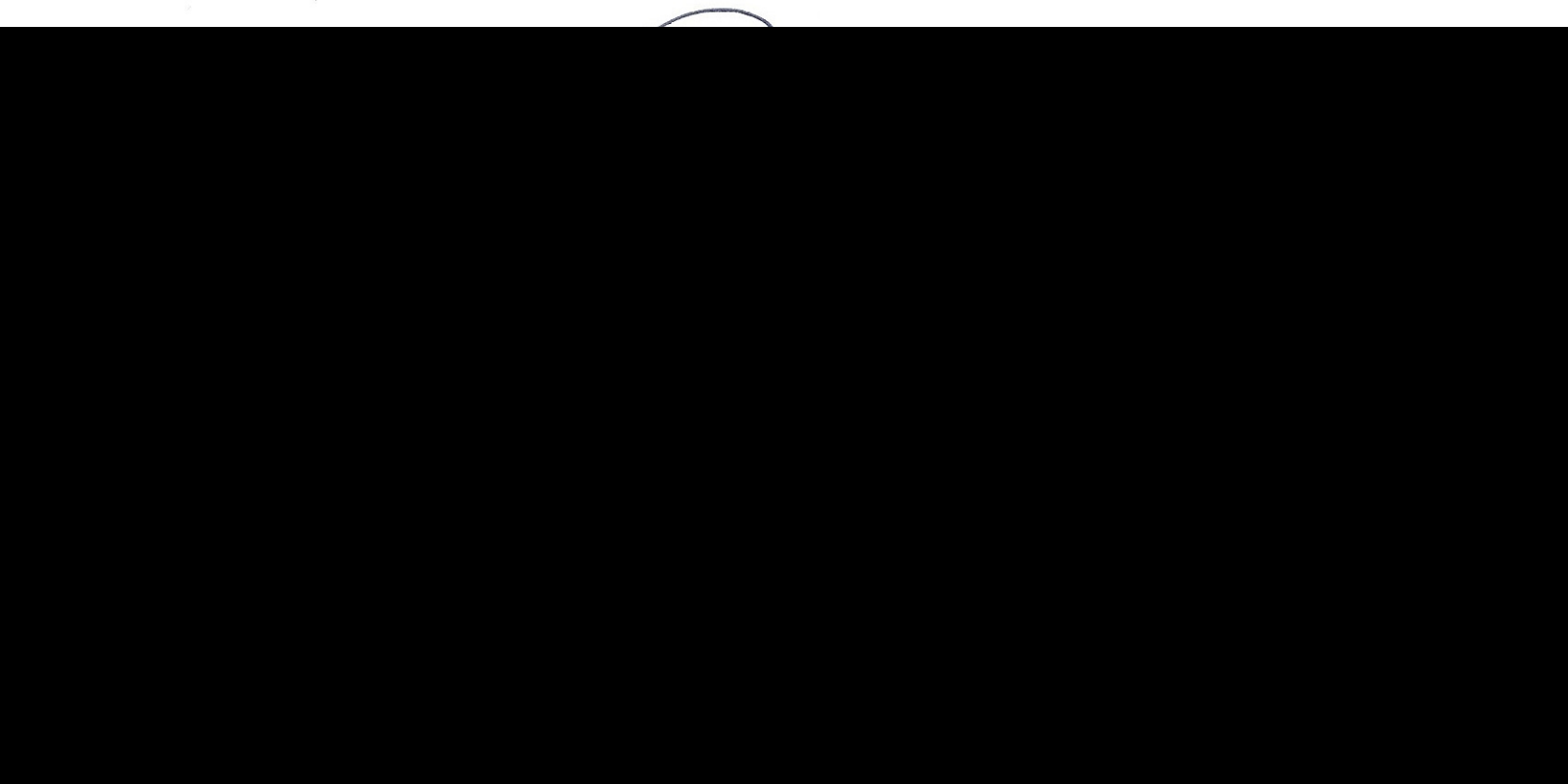

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The text of this dissertation is partially i) a reprint of a manuscript published in *Gene Expression Patterns* (Berberoglu et al., 2009), and ii) a manuscript in preparation for submission. Su Guo directed and supervised the research that forms the basis of the dissertation.

The author contributions are as follows:

i) Gene Expression Patterns 2009 (Berberoglu et al., 2009) (Chapter 1): Michael A. Berberoglu and Su Guo designed experiments. Michael A. Berberoglu carried out histological experiments, imaging, and data analysis. Zhiqiang Dong created the *fezf2*-GFP transgenic zebrafish line. Thomas Mueller helped with anatomical designations and schematic diagram in the final figure. Michael A. Berberoglu and Su Guo wrote the manuscript.

ii) Manuscript in preparation (Chapter 2): Michael A. Berberoglu and Su Guo designed experiments. Zhiqiang Dong performed embryonic transplantations. Michael A. Berberoglu carried out all other experiments and analyzed data. Michael A. Berberoglu wrote a draft of the manuscript.

This work is comparable with a standard thesis awarded by the University of California, San Francisco.

Understanding the role of *fezf2* in adult neural stem cell maintenance and fate: A study in zebrafish

Michael A. Berberoglu

Abstract

Adult neurogenesis, or the birth of new neurons in the mature brain, is a process that occurs continuously and robustly from neural stem cells located in two discreet regions of the adult mammalian brain, the subventricular zone (SVZ) of the lateral ventricles, and the subgranular zone (SGZ) of the dentate gyrus region of the hippocampus. Though some progress has been made in understanding the factors which regulate the maintenance and fate of adult neural stem cells, our understanding remains limited. Fezf2, a conserved forebrain-specific transcription factor, is expressed during development in both zebrafish and mouse in regions where neural progenitor cells are present, and previous studies point to a role for Fezf2 in embryonic neurogenesis in both systems. Here, we show that *fezf2* is expressed in the adult zebrafish forebrain, most notably in radial glial-like cells of the telencephalic ventricular zone, which label with markers of neural stem cells and proliferation. Further analysis using a *fezf2*-GFP transgenic zebrafish line indicates that these *fezf2*-expressing cells have neural stem cell-like properties, as they are able to self-renew and can likely give rise to glutamatergic neurons in the adult zebrafish telencephalon. Analysis of *too few* (Fezf2) homozygous mutant zebrafish crossed to our *fezf2*-GFP reporter line reveals a previously unreported adult telencephalic phenotype, including an increase in proliferation of *fezf2*-GFP+ cells, and an increase in adult neurogenesis. No major differences are observed in the mutant

telencephalon at early larval stage, suggesting that the phenotype occurs largely at the late-larval to adult stage. Moreover, transplantation of *fezf2*-GFP+ mutant cells into wiltype hosts reveals a cell-autonomous role for *fezf2* in maintaining the non-proliferative state of telencephalic radial glial cells. Taken together, our findings suggest a role for *fezf2* in regulating the proliferation and differentiation of neural progenitor cells in the adult vertebrate telencephalon.

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Introduction

Chapter 1 *(Gene Expr. Patterns 2009)*

Chapter 2

Introduction

Adult neural stem cells and adult neurogenesis in mammals

The continuous production of new neurons in the adult brain, termed adult neurogenesis, was greatly debated when it was first proposed by Altman and colleagues in the 1960s (Altman and Das, 1966). This challenged the traditional view that the central nervous system (CNS) is hard-wired during development, leaving only the possibility for changes, or plasticity, to occur at the level of the synapse rather than the production of new neurons themselves.

Though the field of adult neurogenesis lay dormant for some time, the notion that some new neurons continue to be produced in the adult was later revisited. It is now well established that neurogenesis occurs in the adult mammalian brain, and is most robust in two specific areas. One of these areas is the subventricular zone (SVZ) of the lateral ventricles, where adult neural stem cells give rise to new neurons of the olfactory bulb (OB) by way of migration through the rostral migratory stream (RMS) (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch et al., 1999; Gage et al., 2000; Garcia et al., 2004; Goldman, 2003; Merkle et al., 2004; Taupin and Gage, 2002). The other location of active adult neurogenesis is within the dentate gyrus of the hippocampus, where neural progenitor cells of the subgranular zone (SGZ) give rise to new neurons that migrate a short distance to differentiate in the granule cell layer of the hippocampus (see above references and Zhao et al., 2008) (**Fig.1**).

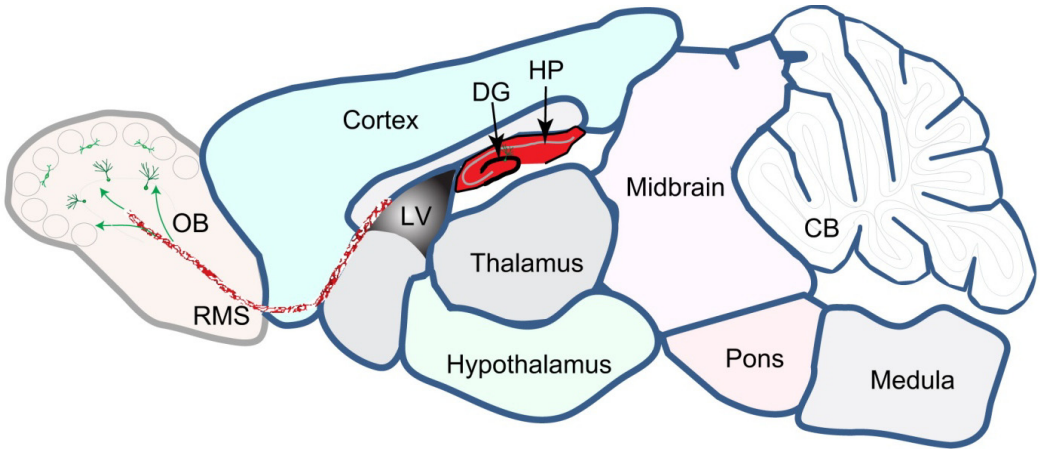

Figure 1. Saggital view of adult rodent brain (schematic drawing) depicting location of adult neural stem cells within the subventricular zone (SVZ) of the lateral ventricles, which give rise to neurons of the olfactory bulb, and neural progenitor cells within the dentate gyrus region of the hippocampus. *(Ming and Song., 2011)*

Why are we interested in studying adult neurogenesis?

There is great interest in understanding the functional role of these new neurons. It is an important question as to why an organism would retain such processes (including primary progenitors, migration, and replacement of neurons) if these neurons do not serve any purpose. A majority of previous research on adult neurogenesis has focused on the hippocampus region, revealing roles for these new-born adult neurons in learning and memory (reviewed in Zhao et al., 2008). Other studies have suggested a correlation between aberrant adult neurogenesis and affective disorders including anxiety and depression (reviewed in Zhao et al., 2008). Furthermore, reports have suggested that new OB neurons may be important for social recognition that is dependent on olfaction, or smell (reviewed in Zhao et al., 2008). These new neurons are thus likely to be playing important roles in the adult brain. Additionally, the study of adult neurogenesis is critical to better understand the regenerative capacity of the adult CNS, and also to understand the mechanisms that control neural stem cell behavior, which can provide novel insights

for the development of stem cell replacement therapies aimed at repairing diseased brain tissue and neurodegeneration.

The zebrafish as a model system

Over the past several years, the zebrafish has emerged as a new and comparable vertebrate model system to study adult neurogenesis. A look into the adult zebrafish telencephalon reveals proliferating cells throughout the ventricular zone (VZ) that can be compared with neural stem cells of the adult mouse telencephalon, especially those within the SVZ (Chapouton et al., 2007). Though the overall architectural layout of the adult zebrafish brain is different from that of mouse, the telencephalon as a brain region is conserved, albeit with a smaller size and distinct location (Chapouton et al., 2007). During development, the zebrafish telencephalon goes through a process known as eversion (Wullimann and Puelles, 1999), and it is for this reason that the ventricular zone wraps around the outside of the telencephalon, where proliferative cells are found (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006) (**Fig. 2**). As in mouse, BrdU pulse-chase studies in zebrafish suggest that neural progenitor cells of the adult zebrafish telencephalon contribute to new neurons of the olfactory bulb (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006).

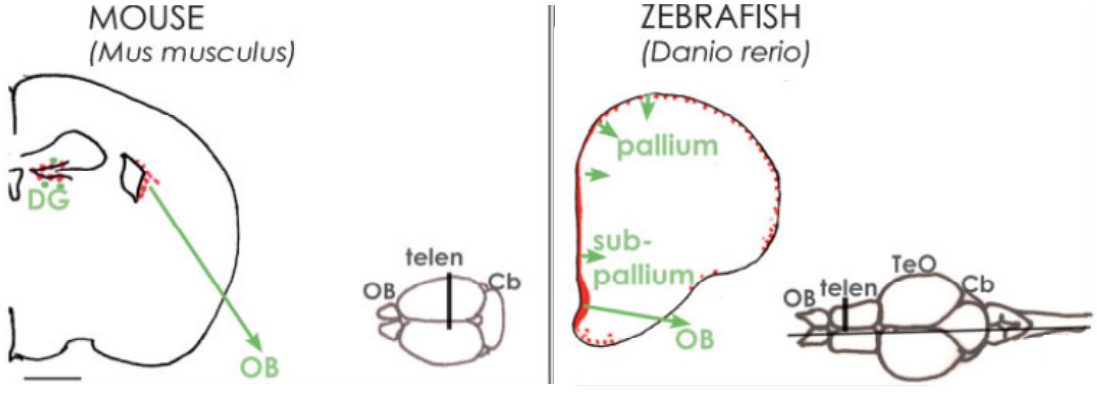

Figure 2. Side-by-side comparison of the adult mouse telencephalon compared with that of the adult zebrafish, with proliferating cells indicated in red. Proliferating cells are present within the adult zebrafish telencephalon, which can be compared to proliferating cells of the adult mouse telencephalon. Eversion of the zebrafish telencephalon during development results in a ventricular zone which surrounds the adult telencephalon. *(modified from Chapouton and Bally-Cuif, 2007)*

Despite these similarities, the adult zebrafish brain is also different from the adult mouse brain. As seen in a saggital view of the adult zebrafish brain labeled with PCNA (proliferating cells nuclear antigen; marker of dividing cells) (see **Fig. 3**), proliferating cells are present essentially throughout the entire adult zebrafish brain. These proliferating cells (which include radial glial-like cells as well as intermediate progenitors) are found not only in the telencephalon, but also in other regions including the hypothalamus and cerebellum, among others. Studies are currently investigating the neurogenic potential of these regions in zebrafish, which may display an enhanced ability for adult neurogenesis. As the zebrafish brain continues to grow during adulthood, it is worth noting that enhanced adult neurogenesis in zebrafish may simply be an extension of development. However, the more quiescent nature of progenitor cells within the adult zebrafish brain compared with embryonic stages suggests that the adult brain is different from that of embryonic stages.

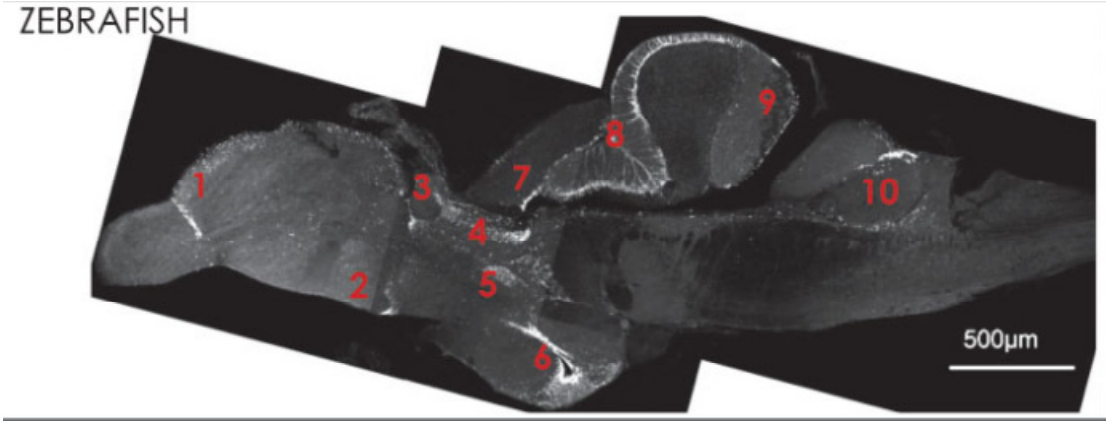

Figure 3. Saggital section through adult zebrafish brain with labeling for the proliferating cell nuclear antigen (PCNA) shows proliferating cells and germinal zones throughout the entire adult zebrafish brain, in regions including the telencephalon, hypothalamus and cerebellum, among others.

(telencephalon (1), preoptic area (2), habenula (3), thalamus (4), posterior tuberculum (5), ventricular domain of the hypothalamus (6), along the torus longitudinalis (7), at the posterior border of the optic tecum at the border between midbrain and hindbrain, and in the cerebellum (valvula cerebelli (8), corpus cerebelli (8), lobus caudalis (9), and facial lobe (10))

(modified from Chapouton and Bally-Cuif, 2007)

It thus appears that the zebrafish has maintained many germinal (and possibly neurogenic) zones that may have been lost in mammals. Whether these zones are used for replacement of cells or for brain growth remains to be determined. Along these lines, the zebrafish is known to possess a remarkable capacity for regeneration; for example, zebrafish can regrow their motor neurons following lesion to the spinal cord (Reimer et al., 2008).

Fezf2 and the regulation of embryonic neurogenesis

Fezf2 (formerly known as fezl) is a conserved, forebrain-specific zinc finger transcription factor. *Fezf2* has been studied in the developing mouse brain, where it is expressed in the ventricular zone at E14.5 (Hirata et al., 2004; Inoue et al., 2004). Analysis of Fezf2 KO mice reveals a loss of layer 5 corticospinal motor neurons, indicating a role for Fezf2 in the specification of these cells (Chen et al., 2005a, 2005b; Molyneaux et al., 2005). *Fezf2* is also expressed in the zebrafish brain, with expression being restricted to the developing forebrain. Fezf2, however, is expressed more broadly during earlier stages of development (last gastrula stage), a time when essentially all cells are progenitor cells (Jeong et al., 2007). Thereafter, *fezf2* expression becomes restricted to fewer cells as development progresses. A zebrafish mutant named *too few* was identified for a

significant reduction in hypothalamic dopamine neurons (Guo et al., 1999), and was later found to contain a mutation in the *fezf2* gene (Levkowitz et al., 2003). This mutant suggests a role for *fezf2* in embryonic neurogenesis in zebrafish as well.

Summary of major findings (for Chapter 1)

Based on the expression of *fezf2* in embryonic progenitor cells as well as our interest in studying adult neural stem cells and adult neurogenesis in zebrafish, we proposed the following hypothesis: *fezf2* is expressed in neural stem cells of the adult zebrafish brain. Using *in situ* hybridization on vibratome sections, we found that *fezf2* is expressed in the adult zebrafish brain, and that its expression is restricted to the forebrain. We were most excited to see expression in the telencephalic ventricular zone. Additionally, expression was detected in the preoptic region and hypothalamus. Using a transgenic line in which the *fezf2* promoter drives expression of GFP, we found that *fezf2*-GFP+ cells of the telencephalic ventricular zone have a characteristic radial glial morphology and label with markers of neural stem cells and proliferation (see Chapter 1, and Berberoglu et al., 2009).

Summary of major findings (for Chapter 2)

In Chapter 2, we wanted to determine whether *fezf2*-expressing radial glial-like cells function as neural stem cells in the adult zebrafish brain, by determining whether they fulfill the defining criteria of a stem cell --- self-renewal, and the ability to give rise to differentiated cell types. As described in detail in Chapter 2, we were able to show that *fezf2*-GFP+ cells have the ability to self-renew, and can likely give rise to neurons in the adult zebrafish telencephalon.

In zebrafish, *fezf2* was expressed most strongly in the midline ventricular zone of the dorsal telencephalon (pallium), anatomically corresponding to the amygdala (Dm; medial part of the dorsal telencephalon). As such, there are important functional implications as to the generation of new neurons within the adult zebrafish amygdala, as well as interest in understanding the type of neurons that are produced in this region at the adult stage. Through BrdU pulse-chase labeling, we have shown that *fezf2*-GFP+ radial glial-like cells may be able to give rise to neurons in the adult zebrafish Dm region. We identified a majority of these neurons as *vglut2a*-containing, indicative of a glutamatergic phenotype (see Chapter 2).

To determine the function of Fezf2 in self-renewal or differentiation of radial glial-like cells of the adult zebrafish telencephalon, we used the *too few* (Fezf2) mutant. Our analysis of the adult *too few* mutant reveals a previously unreported telencephalic phenotype, characterized by a smaller telencephalon, while the rest of the brain appears normal. After crossing this mutant to the *fezf2*-GFP transgenic line, analysis on sections reveals an increase in proliferation of *fezf2*-GFP+ homozygous mutant cells compared with heterozygote sibling controls. We also notice a build-up of neurons in the mutant telencephalon. BrdU pulse-chase studies suggest an increase in neurogenesis in the adult *too few* mutant telencephalon (see Chapter 2). No major differences were observed in the mutant telencephalon at the larval stage, suggesting that the phenotype occurs largely at the late-larval to adult stage.

To determine whether Fezf2 functions autonomously in telencephalic radial glial-like cells to regulate their proliferation, *fezf2*-GFP+ mutant cells were transplanted into wildtype hosts at the embryonic stage, and hosts were grown to the adult stage for

analysis. Our results from these studies indicate a cell-autonomous role for *fezf2* in regulating the proliferation of telencephalic radial glial-like cells. Taken together, our work demonstrates a role for *fezf2* in maintaining the non-proliferative state of radial glial-like progenitor cells in the adult zebrafish telencephalon.

Significance

The work carried out in this dissertation aims to better understand the factors which regulate the maintenance and differentiation of neural progenitor cells, which may provide novel insight for the treatment of neurodegenerative disorders through precisely directed regenerative therapies. Therapies aimed at stimulating endogenous neural stem/progenitor cells to proliferate and give rise to neurons, as well as those in which neural stem cells are injected for the regeneration of neural tissue will be useful avenues for future research.

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CHAPTER 1

fezf2 expression delineates cells with proliferative potential and expressing markers of neural stem cells in the adult zebrafish brain

Michael A. Berberoglu, Zhiqiang Dong, Thomas Mueller, and Su Guo*

Programs in Developmental Biology, Neuroscience and Human Genetics, Department of Bioengineering and Therapeutic Sciences, University of California, San Francisco; San Francisco, CA, 94158-2811, USA.

Author for correspondence: su.guo@ucsf.edu

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Berberoglu, M.A., Dong, Z., Mueller, T., Guo, S. (2009). fezf2 expression delineates cells with proliferative potential and expressing markers of neural stem cells in the adult zebrafish brain. Gene Expr. Patterns 9: 411-422.

Abstract

Fezf2 (also known as Fezl, ZNF312, or Zfp312) is an evolutionarily conserved forebrain-specific zinc finger transcription factor that is expressed during development and is implicated in patterning as well as neurogenesis in both zebrafish and mice. Despite these findings, the expression of *fezf2* in the adult brain has not been well characterized, and *fezf2* function in the adult brain remains unknown. The zebrafish has recently emerged as a new model system to study adult neurogenesis, given its similarity to mammalian systems and enhanced capability of undergoing adult neurogenesis. Through RNA *in situ* hybridization and using a *fezf2* promoter-driven GFP transgenic line, we present data showing that *fezf2* is expressed in radial glial progenitor cells of the telencephalic ventricular zone in the adult zebrafish brain, which co-express markers of neural stem cells and proliferation. Additionally, we identify the preoptic region and the hypothalamus as *fezf2*-expressing neurogenic regions in the adult zebrafish brain, where *fezf2* labels progenitor cells as well as postmitotic neurons. Our findings establish Fezf2 as a novel marker for adult telencephalic ventricular progenitor cells that express markers of neural stem cells in zebrafish and lay a critical foundation for future investigation of Fezf2 function in the maintenance and differentiation of neural stem cells in the adult vertebrate brain.

1. Results and discussion

Neurogenesis, or the birth of new neurons, occurs in the adult vertebrate brain (Chapouton et al., 2007; Zhao et al., 2008). In the adult mammalian brain, cells within the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus have stem cell-like function (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch et al., 1999; Gage et al., 2000; Garcia et al., 2004; Goldman, 2003; Merkle et al., 2004; Taupin and Gage, 2002). Adult neural stem cells (NSCs) of the SVZ give rise to neurons of the olfactory bulb via the rostral migratory stream (RMS), while NSCs of the dentate gyrus give rise to granule neurons that remain in the hippocampus. These adult-born neurons are thought to play important roles in olfaction, learning and memory, as well as in the regulation of affective states (Zhao et al., 2008). Studies have implicated the role of about a handful of transcription factors (such as Sox2) in the regulation of adult NSCs (Zhao et al., 2008; Episkopou, 2005; Ferri et al., 2004). However, our understanding of the factors that regulate the maintenance and/or differentiation of adult NSCs remains limited.

Whereas much of the research to date concerning adult NSCs has focused on rodents, the zebrafish, an organism that is highly amenable to molecular genetic manipulations (Eisen, 1996; Patton and Zon, 2001), has emerged over the past few years as a new model system to study adult neurogenesis (Adolf et al., 2006; Byrd and Brunjes, 1998; Chapouton et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Lam et al., 2009; Zupanc, 2008; Zupanc et al., 2005). These studies have suggested that the adult zebrafish telencephalon, specifically the ventricular zone, contains radial glial cells that label for neural stem cell-specific markers and may thus act as adult NSCs (Chapouton et al.,

2007). Moreover, proliferative zones are found to be present throughout the entire adult zebrafish brain and are not restricted to the SVZ and hippocampus as reported in mammals (Adolf et al., 2006; Chapouton et al., 2006; Chapouton et al., 2007; Grandel et al., 2006). Furthermore, recent studies have shown that the adult zebrafish spinal cord can regenerate following injury (Reimer et al., 2008), a phenomenon that is not observed in the mammalian central nervous system (CNS). Thus, non-mammalian vertebrate species, such as zebrafish, provide a useful model to study adult neurogenesis, and may help us to better understand the regenerative capacity of the vertebrate CNS and uncover new molecular and cellular mechanisms.

Fezf2 (also know as Fezl, ZNF312, or Zfp312) is an evolutionarily conserved forebrain-specific zinc finger transcription factor (Shimizu and Hibi, 2009). In developing zebrafish embryos, *fezf2* is expressed in forebrain progenitor cells during the late gastrula period (~8 hours post fertilization) (Hashimoto et al., 2000; Jeong et al., 2007). As cellular differentiation takes place in the developing forebrain, *fezf2* expression (seen by *in situ* hybridization) becomes rapidly down-regulated but remains in small clusters of cells. Morpholino anti-sense oligonucleotide-based knockdown of *fezf2* leads to alteration in forebrain subdivisions, suggesting a role in brain patterning (Jeong et al. 2007). A zebrafish mutant for *fezf2* (named *too few*) was discovered by screening for defects in dopamine (DA) neuron development (Guo et al., 1999; Levkowitz et al., 2003). Furthermore, studies have revealed an apparent reduction of DA neurons in the adult *too few* mutant brain as well (Rink and Guo, 2004), suggesting a role for Fezf2 in neurogenesis. In developing mouse embryos, *fezf2* is first expressed at E8.5 and is maintained to E14.5, where it is detected in the ventricular zone in a pattern consistent

with the location of neural progenitors (Hirata et al., 2004; Inoue et al., 2004). *fezf2* is involved in the establishment of diencephalic subdivisions (Hirata et al., 2006), and inactivation of Fezf2 leads to defects in the formation of subplate neurons (Hirata et al., 2004). In addition, *fezf2* is critical for the birth and specification of corticospinal motor neurons (Chen et al., 2005a, 2005b; Molyneaux et al., 2005). Together, these findings suggest a conserved role for *fezf2* in forebrain patterning and embryonic neurogenesis.

Here, we report that *fezf2* expression is detected in the adult zebrafish forebrain. In the telencephalon, *fezf2* expression is restricted to the telencephalic ventricular zone. Additionally, *fezf2* is expressed in the diencephalon in both the preoptic region as well as in the hypothalamus. Further analysis using a *fezf2* promoter-driven GFP transgenic line indicates that telencephalic ventricular *fezf2*-expressing cells have radial glial morphology and express markers for neural stem cells and proliferation. *fezf2*-GFP+ progenitor and differentiated cells are present in the olfactory bulb, the preoptic region, and the hypothalamus, which colocalize with markers of neural stem cells and proliferation, or the neuronal marker Hu, suggesting that *fezf2*-GFP+ progenitors may give rise to adult-born neurons that maintain GFP expression or that *fezf2* is expressed in differentiated neurons in these regions.

These results establish Fezf2 as a novel marker for adult telencephalic ventricular progenitor cells in zebrafish. Since these cells express NSC markers, they are likely to be adult neural stem cells. Our results also suggest that in addition to the SVZ-like neurogenic niche of the telencephalon, the preoptic region and hypothalamus are *fezf2*-expressing neurogenic regions in the adult zebrafish brain, where *fezf2* expression is likely to be in both progenitors and differentiated cells. These findings lay a critical

foundation for the use of Fezf2 as a marker to investigate the multipotent and self-renewal capacities of adult neural stem cells in zebrafish and for future investigation of Fezf2 function in the maintenance or differentiation of neural stems cells in the adult vertebrate brain.

1.1 fezf2 mRNA is detected in the adult zebrafish brain

Since the expression of *fezf2* in the adult zebrafish brain has not previously been characterized, we performed *in situ* hybridization for *fezf2* on 100μM Vibratome sections of the wildtype adult zebrafish brain. Our results indicate that *fezf2* expression is restricted to the forebrain, most notably in the telencephalic ventricular zone and also in the olfactory bulb, preoptic region, and hypothalamus (Figure 1) (anatomical designations according to Wullimann et al., 1996).

In the olfactory bulb, *fezf2* expression is detected caudally in the periphery but is absent from the internal layers (Figure 1B, arrowhead). Within the telencephalon, strong expression is detected in the midline region of the pallial (D, dorsal telencephalic) ventricular zone (VZ), but seems to be largely absent from the subpallial (V, ventral telencephalic) ventricular zone. Although *fezf2* expression has not been reported to be expressed in the subpallial region of the telencephalon in either embryonic zebrafish or mouse (Hashimoto et al., 2000; Hirata et al., 2004; Inoue et al., 2004; Jeong et al., 2007), we cannot exclude the possibility that there may be an extension of *fezf2* expression into the Vd region of the subpallium in the adult zebrafish brain (Figure 1C). *fezf2* expression is also detected bilaterally on the ventral side of this telencephalic section in Figure 1C (arrowhead). These may be *fezf2*-expressing cells of the caudal olfactory bulb (given the plane of section), or perhaps another area expressing *fezf2* in the forebrain. It is also possible that this expression may represent the ventral subpallial stripe that is thought to be reminiscent of the RMS in mammals (Adolf et al., 2006). In the diencephalon, *fezf2* is strongly expressed in both ventricular and adjacent non-ventricular cells of the preoptic

region (Po) and the caudal hypothalamus (Hc) (Figure 1D, E). *fezf2* expression is also detected in the PVO region of the posterior tuberculum (Figure 1E).

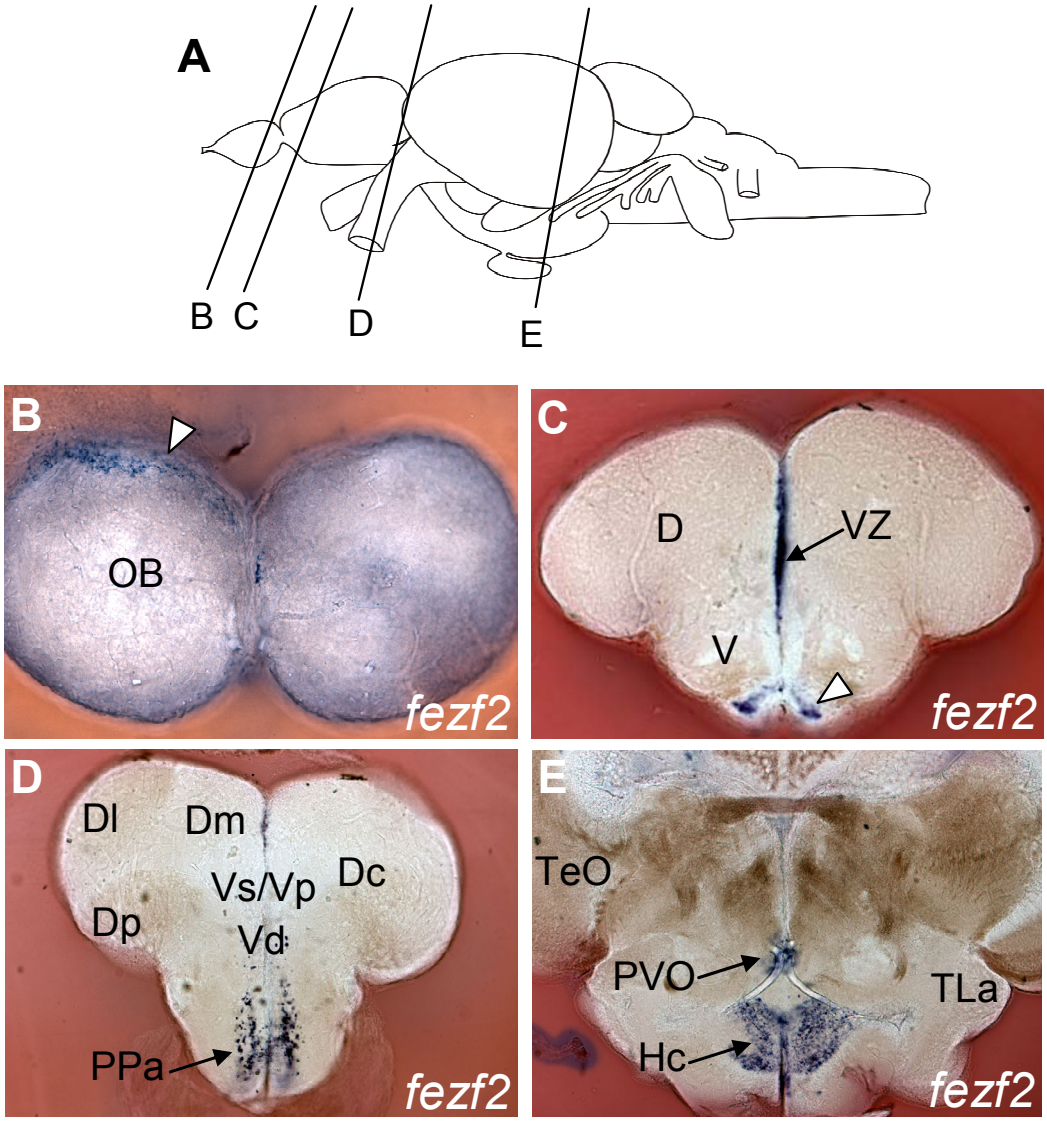


Figure 1. *fezf2* mRNA expression in the adult zebrafish brain by *in situ* hybridization.
(A) Schematic of adult zebrafish brain showing plane of section in B-E. (B) Coronal section through caudal olfactory bulb showing *fezf2* expression in the periphery (arrowhead) (10X magnification). (C) Coronal section through telencephalon showing strong *fezf2* expression in the pallial ventricular zone (midline), as well as bilateral ventral expression (arrowhead) (10X magnification). (D) Coronal section showing *fezf2* expression in the preoptic region (10X magnification). (E) Coronal section showing *fezf2* expression in the hypothalamus (10X magnification). Schematic modified from Wullimann et al., 1996. Abbreviations: **OB**

olfactory bulb; **D** dorsal telencephalon; **V** ventral telencephalon; **VZ** ventricular zone; **Dl** lateral zone of D; **Dm** medial zone of D; **Dc** central zone of D; **Dp** posterior zone of D; **Vs** supracommissural nucleus of V; **Vp** postcommissural nucleus of V; **Vd** dorsal nucleus of V; **Po** preoptic region; **TeO** tectum opticum; **PVO** paraventricular organ; **Hc** caudal hypothalamus; **TLa** torus lateralis.

1.2 fezf2-GFP transgenic line

To further investigate the identity of these *fezf2*-expressing cells, we created a *fezf2* promoter-driven GFP transgenic line, using 5' and 3' regulatory elements adjacent to the *fezf2* gene. As shown in Figure 2, the *fezf2*-GFP transgenic line drives reporter expression in a similar pattern as *fezf2* transcripts. In the olfactory bulb, GFP expression is detected caudally in the periphery (Figure 2B) and is more broadly expressed than *fezf2* transcripts in this area (Figure 1B, arrowhead). Notably, some GFP+ cells are detected within the more internal layers of the olfactory bulb (Figure 2B, arrowheads), which were not seen by *in situ* hybridization for *fezf2*. These cells may be recently migrated cells from either the telencephalon (although we do not detect GFP-expressing cells in the ventral subpallial stripe) or from local *fezf2*-GFP+ progenitors within the olfactory bulb, which have retained GFP expression. It is also possible that *fezf2* is expressed at a very low level in these cells and is therefore not detected by *in situ* hybridization, or that these cells may express GFP ectopically due to a lack of certain repressive elements in the *fezf2* promoter used to drive GFP.

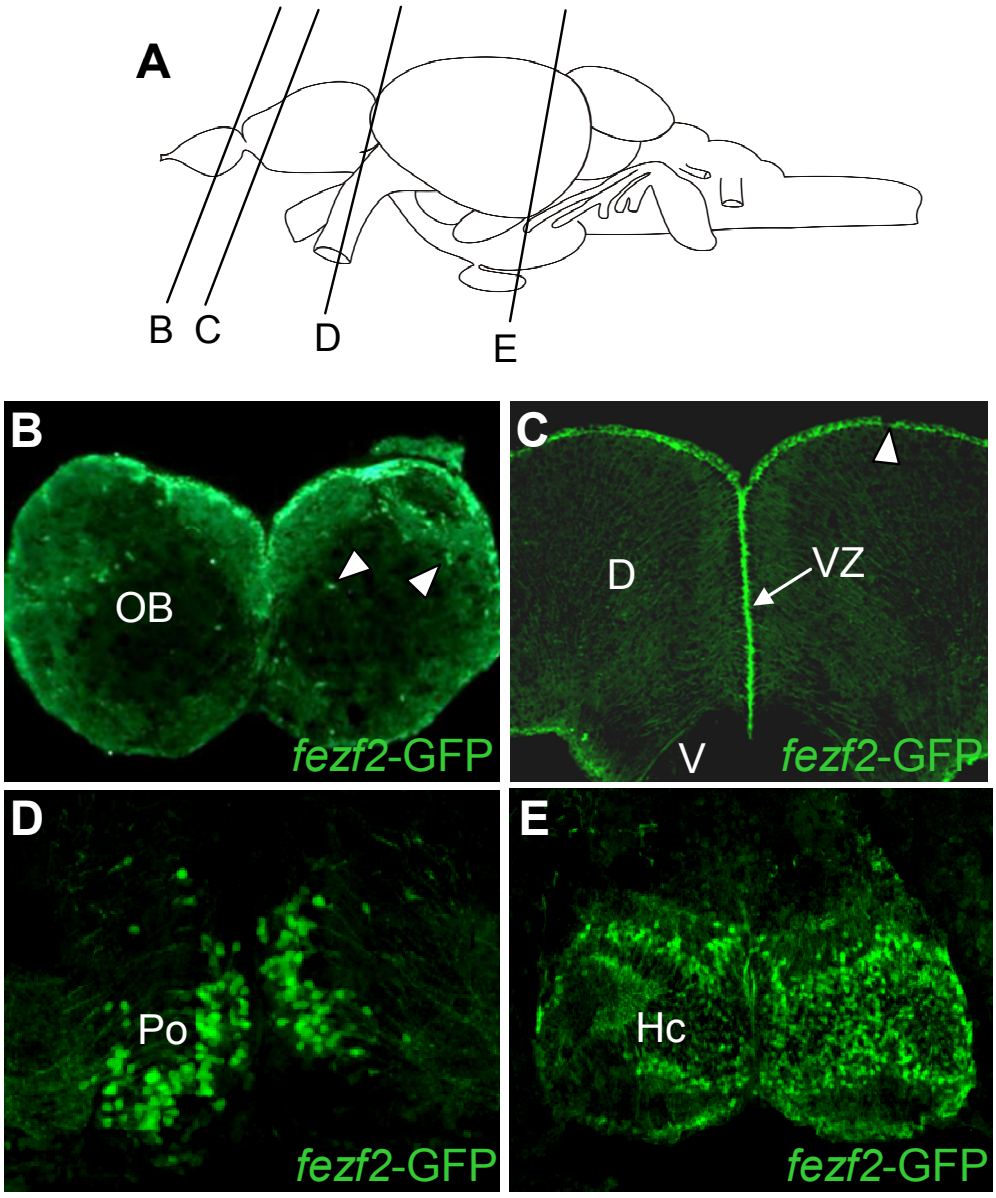


Figure 2. *fezf2*-GFP transgenic line drives reporter expression in a similar pattern as *fezf2* transcripts. (A) Schematic of adult zebrafish brain showing plane of section in B-E. (B) Coronal section through caudal olfactory bulb showing *fezf2* expression broadly in the periphery as well as additional *fezf2-GFP+* cells in the more internal layers (arrowheads) (20X magnification). (C) Coronal section through telencephalon showing *fezf2* expression in the pallial ventricular zone (20X magnification). Note that GFP expression is also observed at the dorsal telencephalic ventricular surface (arrowhead), which is not seen with *fezf2* mRNA expression. (D) Coronal section showing *fezf2* expression in the preoptic region (40X magnification). (E) Coronal section showing *fezf2* expression in the hypothalamus (40X magnification). Schematic modified from Wullimann et al., 1996. Abbreviations: **OB**

olfactory bulb; **D** dorsal telencephalon; **V** ventral telencephalon; **VZ** ventricular zone; **Po** preoptic region; **Hc** caudal hypothalamus.

In the telencephalon, GFP expression is restricted to pallial (D, dorsal telencephalic) ventricular zones (although we cannot exclude the possibility that *fezf2*-GFP expression may extend into the Vd region of the subpallium) (Figure 2C), similar to *fezf2* transcript distribution. In addition, GFP expression is detected in the dorsal proliferative zone of the pallium (Figure 2C, arrowhead) and in some sparse postmitotic cells within the gray matter of the subpallium (Figure 3H), which is not observed by *in situ* hybridization for *fezf2* (Figure 1C). These GFP-positive/Hu+ postmitotic cells may represent newly-born neurons within the adult telencephalon, or may express *fezf2* at very low levels not detected by *in situ* hybridization. Alternatively, they may express GFP ectopically due to a lack of certain repressive elements in the *fezf2* promoter used to drive GFP.

Consistent with *fezf2* transcript distribution, GFP expression is also detected in the preoptic region (Po) (Figure 2D) and caudal hypothalamus (Hc) (Figure 2E), as well as in the posterior tuberculum (TPp and PVO) (data not shown; see Figure 7). In the posterior tuberculum, GFP+ cells show almost no overlap with markers of NSCs (e.g. GFAP), proliferation (e.g. PCNA), and young neurons (Hu). These results suggest that *fezf2*-GFP+ cells in the posterior tuberculum may represent mostly mature neurons that no longer express Hu, which largely marks newly born differentiating neurons.

In the adult mouse forebrain, *fezf2* expression is reported to be mainly in differentiated cortical projection neurons, such as the corticospinal motor neurons (Inoue et al., 2004). However, we do not find any *fezf2*-GFP-positive telencephalic projection neurons in adult zebrafish, suggesting that zebrafish do not have corticospinal motor

neurons, or that *fezf2* is not expressed in zebrafish corticospinal motor neurons. Correspondingly, we do not detect *fezf2* mRNA expression in the pallium outside of the ventricular zone, where corticospinal motor neurons would most likely be located.

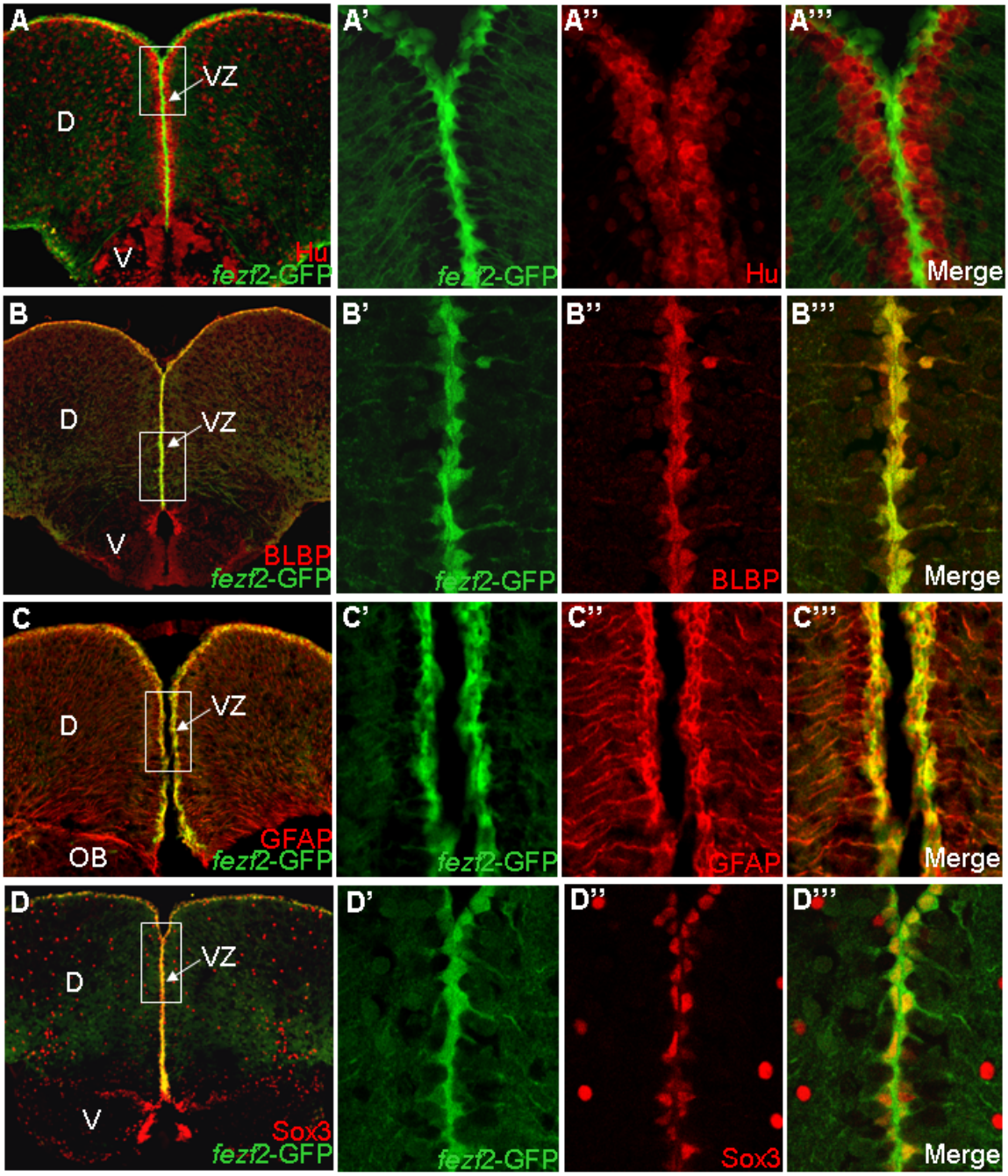
Taken together, *fezf2*-GFP expression largely recapitulates the expression pattern of endogenous *fezf2*.

1.3 fezf2-GFP is detected in radial glial cells of the telencephalic ventricular zone

Upon closer analysis of *fezf2*-GFP+ cells of the telencephalic ventricular zone, we observed that these cells have radial glial morphology and are adjacent to but do not overlap with neurons marked by HuC/D (Figure 3A). Radial glial cells in the adult zebrafish telencephalon have previously been described and label with markers of proliferation (such as PCNA and BrdU) as well as markers of neural stem cells (such as GFAP, BLBP, and Nestin) (Adolf et al., 2006; Grandel et al., 2006; Lam et al., 2009). These radial glial ventricular progenitor cells are thought to function as adult neural stem cells in the zebrafish brain, although their self-renewal and multipotency capabilities are yet to be demonstrated.

Further analysis indicates that these *fezf2*-GFP+ cells colocalize with neural stem cell markers BLBP (brain lipid binding protein), GFAP (glial fibrillary acidic protein), and Sox3 (Figure 3B, C, D). The markers BLBP and GFAP label astrocytic (radial) neural stem cells as well as differentiated astrocytes; however, no differentiated astrocytes are detected with these markers in the adult zebrafish telencephalon. Sox3 marks neural stem cells as well as some postmitotic neurons in mice (Wang et al., 2006). Accordingly, we also observe Sox3-labeled cells throughout the pallium, which colocalize with neuronal

marker HuC/D (data not shown). Our results thus establish Fezf2 as a novel marker for these radial glial telencephalic ventricular progenitor cells.



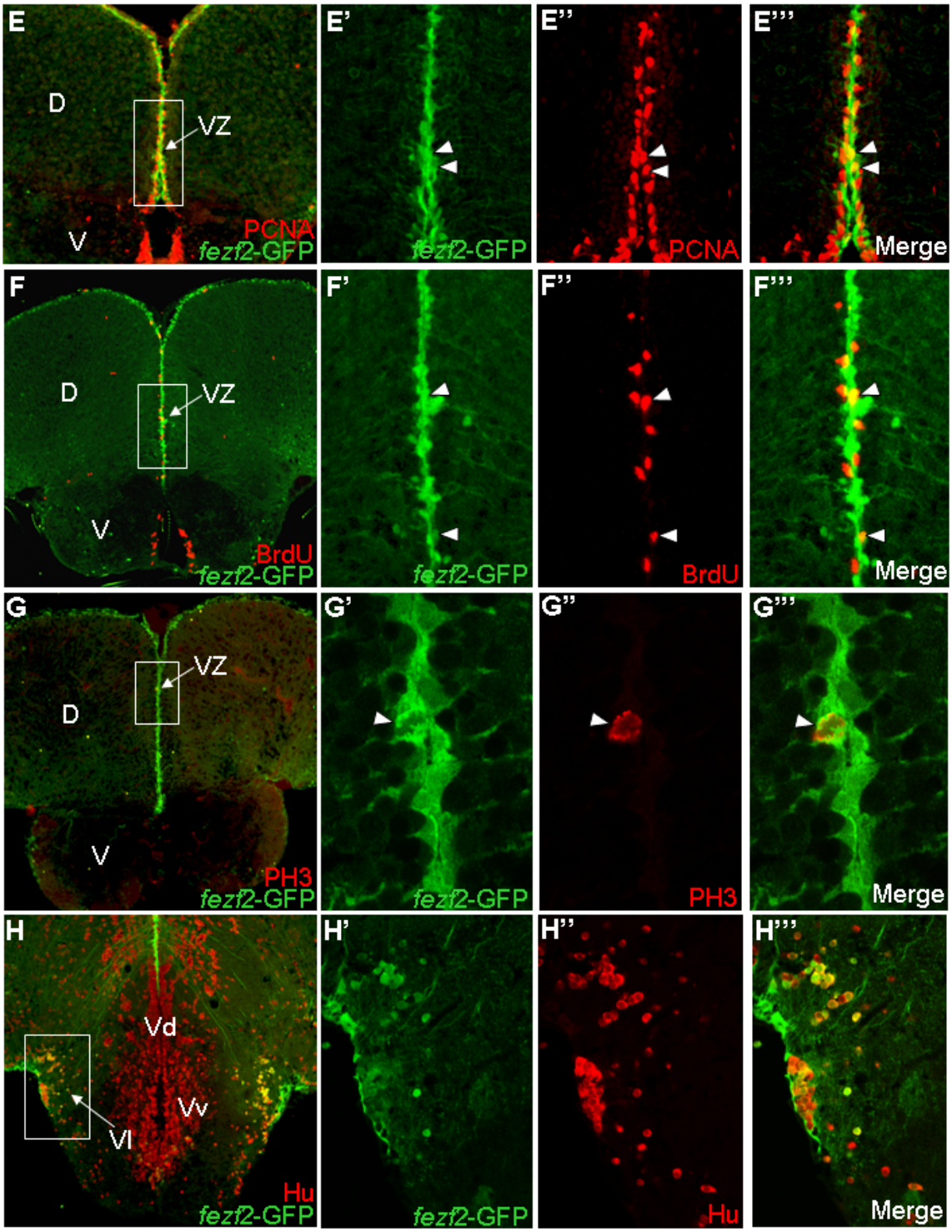


Figure 3. *fezf2* is expressed in radial glial cells of the telencephalic ventricular zone, which colocalize with markers of neural stem cells and proliferation. (A) Coronal section through telencephalon showing double-label of *fezf2*-GFP (green) and Hu (red) (20X magnification). (A'-A''') Closer view of the boxed region (100X magnification) shows that *fezf2*-GFP+ cells

have radial glial morphology (A') and do not overlap with neuronal marker HuC/D (A'''). (B) Coronal section through telencephalon showing colocalization of *fezf2*-GFP (green) and BLBP (red) in the pallial ventricular zone (20X magnification). (B'-B''') Single confocal Z-section (~0.5 micron) of the boxed region (100X magnification) shows that *fezf2*-GFP expression colocalizes precisely with BLBP+ radial glial cells. (C) Coronal section through anterior telencephalon showing double-label of *fezf2*-GFP (green) and GFAP (red) (20X magnification). (C'-C''') Single confocal Z-section of the boxed region (40X magnification) shows that *fezf2*-GFP+ cells colocalize with neural stem cell marker GFAP. (D) Coronal section through telencephalon showing colocalization of *fezf2*-GFP (green) and Sox3 (red) in the pallial ventricular zone (20X magnification). (D'-D''') Single confocal Z-section of the boxed region shows that *fezf2*-GFP+ radial glial cells colocalize with neural stem cell marker Sox3 (100X magnification). (E) Coronal section through telencephalon showing double-label of *fezf2*-GFP and proliferation marker PCNA (20X magnification). (E'-E''') Single confocal Z-section of the boxed region shows that some *fezf2*-GFP+ cells colocalize with PCNA (arrowheads) (40X magnification). (F) Coronal section through telencephalon showing double-label of *fezf2*-GFP and BrdU (Bromodeoxyuridine, S-phase marker) (20X magnification). (F'-F''') Single confocal Z-section of the boxed region shows that some *fezf2*-GFP+ cells colocalize with BrdU (arrowheads) (40X magnification). (G) Coronal section through telencephalon showing double-label of *fezf2*-GFP and PH3 (phospho-histone H3; marker of mitosis) (20X magnification). (G'-G''') Single confocal Z-section of the boxed region shows colocalization of a *fezf2*-GFP+ cell with PH3 (arrowhead) (100X magnification). (H) Coronal section through telencephalon showing colocalization of *fezf2*-GFP with Hu (neuronal marker) in the subpallium (Vl region) (20X magnification). (H'-H''') Single confocal Z-section of the boxed region shows colocalization of *fezf2*-GFP with Hu in the Vl region (40X magnification). Abbreviations: **D** dorsal telencephalon; **V** ventral telencephalon; **VZ** ventricular zone; **OB** olfactory bulb; **Vd** dorsal nucleus of V; **Vl** lateral nucleus of V; **Vv** ventral nucleus of V.

To determine whether these *fezf2*-GFP+ cells have proliferative potential, we used the marker PCNA (proliferating cell nuclear antigen), which labels cells that are actively cycling (Mueller and Wullimann, 2002; Wullimann and Puelles, 1999). We found that some *fezf2*-expressing cells colocalize with PCNA (Figure 3E), suggesting that these cells have proliferative potential. Since a number of *fezf2*-expressing ventricular progenitor cells do not label with PCNA, our results also suggest that these *fezf2*-expressing cells

represent a progenitor population that is quiescent and slow-dividing. It is also possible that some of these *fezf2*-GFP+/PCNA- radial glial-like cells may represent ependymal cells. Ependymal cells have also been shown to label with markers such as GFAP and BLBP in mammals, and recent studies suggest that ependymal cells are not postmitotic but may actually be quiescent adult neural stem cells (Coskun et al., 2008). Furthermore, it is worth noting that some pallial PCNA+ cells are *fezf2*-GFP-. Since *fezf2*-GFP+ cells are in complete overlap with neural stem cell markers BLBP and GFAP in the dorsal telencephalic ventricular region (see Figure 3B, C), which label neural stem cells that are relatively quiescent, this observation makes it unlikely that the PCNA+/*fezf2*-GFP- progenitor cells are NSCs. Thus, these PCNA+/*fezf2*-GFP- cells may represent a population of transit amplifying cells that are more rapidly proliferating and have not been well characterized in the adult zebrafish brain.

To further investigate the proliferative status and relative quiescence of these *fezf2*-expressing cells, we performed BrdU incorporation experiments. As shown in Figure 3F, a few *fezf2*-GFP+ cells do colocalize with BrdU (Bromodeoxyuridine, marker of S-phase), suggesting that these cells enter S-phase. Consistent with the relative quiescent nature of these *fezf2*-GFP+ ventricular progenitor cells, only very few colocalize with BrdU (fewer than with PCNA). Finally, we performed staining with phospho-histone H3 (PH3, a marker of mitosis). As shown in Figure 3G, a *fezf2*-GFP+ cell colocalizes with PH3. Since the M-phase is even shorter than S-phase, *fezf2*-GFP+/PH3+ cells are even more rare than *fezf2*-GFP+/BrdU+ cells.

Previous studies in the adult zebrafish brain using PCNA and bromodeoxyuridine (BrdU, marker of S phase) have suggested that the pallial ventricular progenitor cells (as

well as those in the Vd region of the subpallium) are generally more slowly dividing compared with those of the subpallium (Adolf et al., 2006; Chapouton et al., 2007; Grandel et al., 2006; Lam et al., 2009). As our results indicate that *fezf2*-expressing cells are located in the pallial ventricular zone (as well as potentially in Vd), these cells are likely to represent relatively quiescent adult neural stem cells.

1.4 fezf2-GFP in the olfactory bulb

In the olfactory bulb, *fezf2*-GFP is expressed broadly in the periphery of the most caudal part of the olfactory bulb, as well as sparsely in the more internal layers (Figure 2B). *fezf2*-GFP+ cells seem to colocalize with neural stem cell markers BLBP and GFAP (Figure 4A, B). *fezf2*-GFP+ cells in this area also colocalize with PCNA, suggesting that these cells have proliferative potential (Figure 4C). BrdU incorporation studies further confirm the proliferating status of these *fezf2*-expressing cells of the caudal olfactory bulb (Figure 4D). These results suggest that *fezf2*-expressing cells of the olfactory bulb may represent local neural progenitor cells. Interestingly, we also observe some *fezf2*-GFP+ cells that colocalize with neuronal marker HuC/D (Figure 4E). These may be newly born neurons that are derived from local progenitor cells within the olfactory bulb and may include projection neurons such as mitral and/or tufted cells. Alternatively, *fezf2* may be expressed at a very low level in these cells and is not detected by *in situ* hybridization. It is also possible that these cells may express GFP ectopically due to a lack of certain repressive elements in the *fezf2* promoter used to drive GFP.

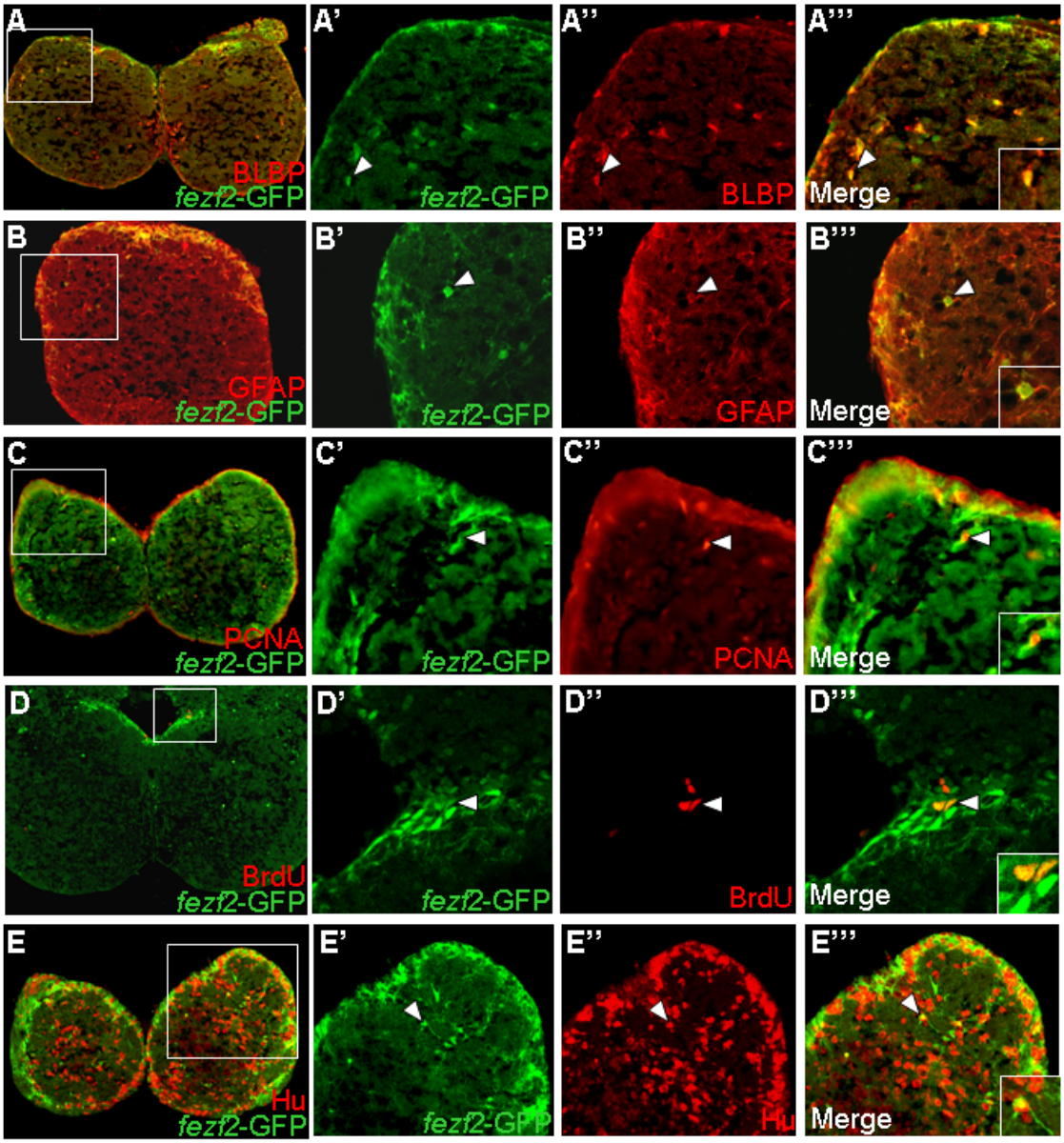


Figure 4. *fezf2*-GFP+ cells colocalize with markers of neurons and proliferation in the olfactory bulb. (A) Coronal section through the olfactory bulb showing double-labeling of *fezf2*-GFP (green) and BLBP (red) (20X magnification). (A'-A''') Some *fezf2*-GFP+ cells colocalize with neural stem cell/astrocyte marker BLBP (arrowhead), suggesting that these are either local neural stem cells or differentiated astrocytes within the olfactory bulb (40X magnification). (A''') Inset shows colocalization in a single cell at higher magnification. (B) Coronal section showing *fezf2*-GFP (green) and GFAP (red) labeling in a single olfactory bulb. (B'-B''') Some *fezf2*-GFP+ cells colocalize with GFAP (arrowhead), suggesting that these are either local neural stem cells or differentiated astrocytes (40X magnification). (B''') Inset shows colocalization in a single cell at higher magnification. (C) Coronal section

showing double-labeling of *fezf2*-GFP (green) and PCNA (red) (20X magnification). (C'-C''') Some *fezf2*-GFP+ cells colocalize with proliferation marker PCNA (arrowhead) (40X magnification). (C''') Inset shows colocalization in a single cell at higher magnification. (D) Coronal section showing double-labeling of *fezf2*-GFP (green) and BrdU (red) in the caudal olfactory bulb (20X magnification). (D'-D''') Some medio-dorsal *fezf2*-GFP+ cells colocalize with BrdU (S-phase marker) (arrowhead) (40X magnification). (D''') Inset shows colocalization at higher magnification. (E) Coronal section showing double-labeling of *fezf2*-GFP (green) and Hu (red) (20X magnification). (E'-E''') Closer view of the boxed region shows that some *fezf2*-GFP+ cells colocalize with neuronal marker Hu (arrowhead) (40X magnification). (E''') Inset shows colocalization in a single cell (depicted by arrowhead) at higher magnification.

The zebrafish telencephalon is known to undergo eversion (Wullimann and Mueller, 2004). Though the olfactory bulb has partial pallial origin, the dorsal region of the olfactory bulb is largely non-everted. Nevertheless, the very caudal portion of the olfactory bulb that attaches to the rest of the telencephalon is ventricular and shows proliferative cells (cross section 50 of Wullimann et al., 1996). It is therefore possible that *fezf2* is expressed in this ventricular progenitor zone (dorsal to the Internal Cell Layer (ICL) and ventral to the anterior-most part of Vd) at the point of attachment of the caudal olfactory bulb to the rest of the telencephalon. This interpretation is consistent with our finding that peripheral *fezf2* mRNA and *fezf2*-GFP expression in the olfactory bulb is localized exclusively to the most caudal areas and is absent from more anterior regions of the olfactory bulb (data not shown).

fezf2 expression has been reported in the vomeronasal organs in the nasal septum of the developing mouse embryo (Hirata et al., 2004). Thus, it is also possible that *fezf2* is expressed in the olfactory sensory or pheromone-sensing neurons in the adult zebrafish, which send axons to the olfactory bulb. *fezf2* mRNA may be transported down the axons of these sensory neurons, which may account for the observed staining by *in situ*

hybridization (Figure 1B). The *fezf2*-GFP signal near the periphery of the olfactory bulb may thus represent the neurites of these sensory neurons.

1.5 fezf2-GFP in the preoptic region and hypothalamus

In the adult mammalian brain, neurogenesis is thought to be restricted to the SVZ and hippocampus (Zhao et al., 2008). In contrast, proliferative zones are more widespread in the adult zebrafish brain (Chapouton et al., 2007), allowing for the identification of new neurogenic regions that may not be present in mammals. Previous BrdU/Hu studies have suggested that the preoptic region and hypothalamus may represent actively proliferating and neurogenic regions in the adult zebrafish brain (Grandel et al., 2006).

Here, we find that some *fezf2*-GFP+ cells of the preoptic region colocalize with BLBP and GFAP (Figure 5 A, B), which are markers of ventricular radial glia or differentiated astrocytes. Since these cells do not have radial glial morphology and are not located near the ventricle, they likely represent differentiated astrocytes. Consistent with this notion, most *fezf2*-GFP+ cells do not colocalize with PCNA (Figure 5C). Moreover, we observe that a majority of *fezf2*-GFP+ cells colocalize with the neuronal marker HuC/D (Figure 5D), suggesting that *fezf2* is expressed in postmitotic neurons in the preoptic region.

Our results suggest that the adult preoptic region expresses *fezf2* and thus may be dependent on *fezf2* function. This adult neurogenic region may be specific to the adult zebrafish brain, as there have been no reports of adult proliferation within the mammalian preoptic nuclei. Such regions may allow us to better understand the regenerative capacity of the adult CNS and may shed light on cell-intrinsic and extrinsic (i.e. niche) factors that

may be responsible for the increased capacity for regeneration observed in the adult zebrafish CNS.

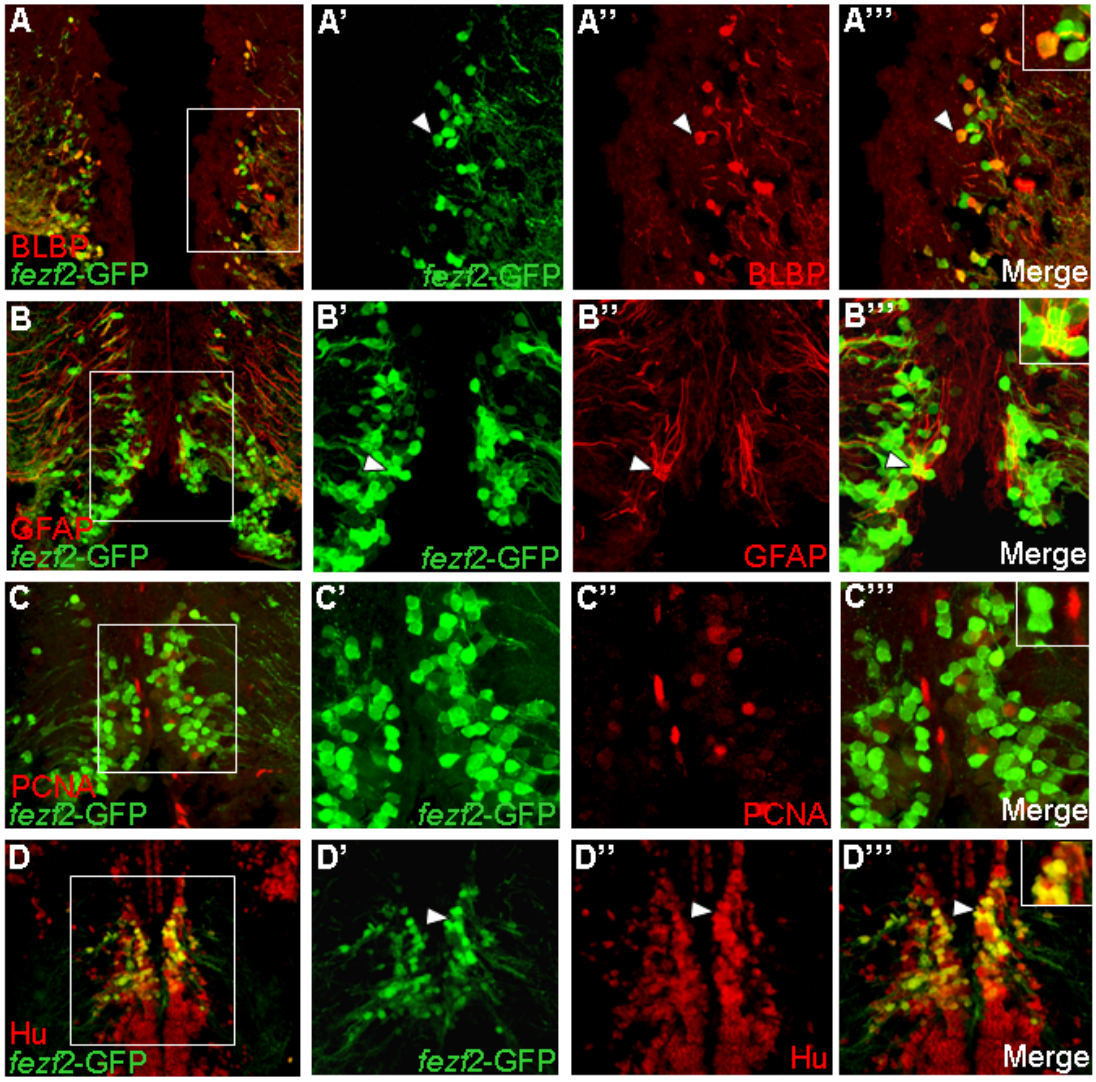


Figure 5. *fezf2*-GFP is expressed largely in postmitotic neurons of the preoptic region. (A) Coronal section through preoptic region showing double-labeling of *fezf2*-GFP (green) and BLBP (red) (40X magnification). (A'-A''') Closer view of the boxed region shows that some *fezf2*-GFP+ cells colocalize with neural stem cell/astrocytic marker BLBP (arrowhead). (A''') Inset shows colocalization in a single cell (depicted by arrowhead) at higher magnification. (B) Double-labeling of *fezf2*-GFP (green) and neural stem cell/astrocytic marker GFAP (red) (40X magnification). (B'-B''') Closer view of boxed region shows colocalization in some cells (arrowhead). (B''') Inset shows colocalization in some cells at higher magnification. (C) Double-labeling of *fezf2*-GFP and proliferating cell marker PCNA

(40X magnification). (C'-C''') Closer view of the boxed region shows that *fezf2*-GFP+ cells do not co-localize with PCNA. (C''') Inset shows lack of colocalization at higher magnification. (D) Double-labeling of *fezf2*-GFP and neuronal marker Hu (40X magnification). (D'-D''') Closer view of boxed region shows a number of *fezf2*-GFP+ cells that colocalize with Hu (arrowhead). (D''') Inset shows colocalization in cells at higher magnification.

Our studies also reveal that the hypothalamus may represent another *fezf2*-expressing neurogenic region in the adult vertebrate brain that has not yet been fully appreciated. Previous studies in mice have identified proliferating cells in the adult hypothalamus that likely give rise to new neurons and glial cells and may play an important role in energy balance (Kokoeva et al., 2005; Kokoeva et al., 2007); however, the cellular and molecular characteristics of these progenitor cells have not been investigated. We find that some *fezf2*-GFP+ cells of the hypothalamus colocalize with neural stem cell markers BLBP and GFAP (Figure 6A, B), as well as the proliferation marker PCNA (Figure 6C). Notably, some *fezf2*-GFP+/BLBP+ cells appear to have radial glial morphology (Figure 6A). It appears that these *fezf2*-expressing radial glial-like cells are located in the PR (posterior recess of diencephalic ventricle) (cross section 173 of Wullimann et al., 1996). Further analysis shows that a number of *fezf2*-GFP+ cells of the hypothalamus also colocalize with the neuronal marker HuC/D (Figure 6D). These findings suggest that the hypothalamus represents an additional conserved region of active neurogenesis in the adult vertebrate brain that may be dependent on Fezf2 function.

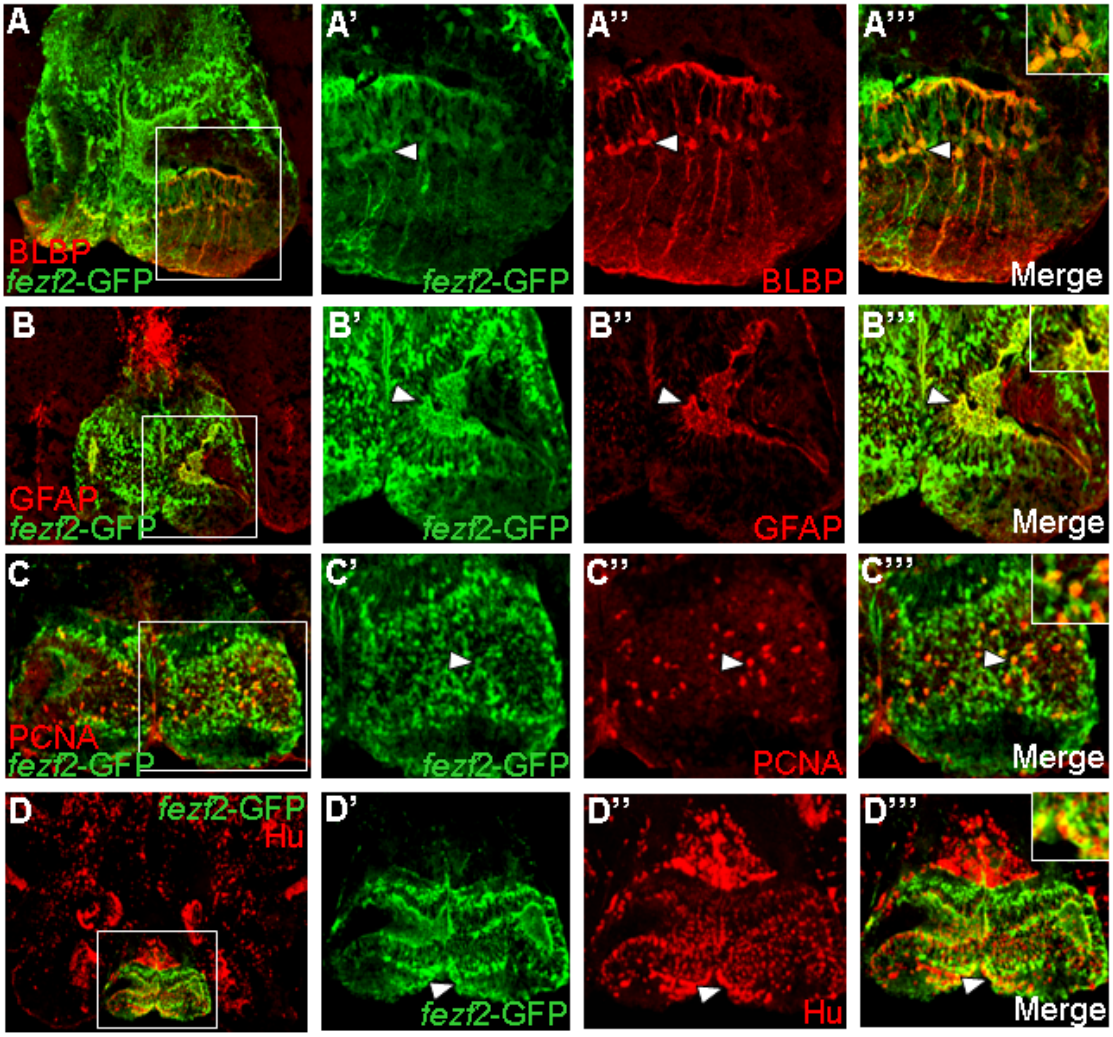


Figure 6. *fezf2*-GFP expression suggests adult neurogenesis in the caudal hypothalamus. (A) Coronal section through hypothalamus showing double-labeling of *fezf2*-GFP (green) and BLBP (red) (40X magnification). (A'-A''') Closer view of the boxed region shows that some *fezf2*-GFP+ cells colocalize with neural stem cell marker BLBP and have radial glial morphology (arrowhead). (A''') Inset shows colocalization in cells (depicted by arrowhead) at higher magnification. (B) Double-labeling of *fezf2*-GFP (green) and neural stem cell marker GFAP (red) (40X magnification). (B'-B''') Closer view of boxed region shows colocalization in some cells (arrowhead). (B''') Inset shows colocalization in cells at higher magnification. (C) Double-labeling of *fezf2*-GFP and proliferating cell marker PCNA (40X magnification). (C'-C''') Closer view of boxed region shows colocalization in some cells (arrowhead). (C''') Inset shows colocalization in cells at higher magnification. (D) Double-labeling of *fezf2*-GFP and neuronal marker Hu (20X magnification). (D'-D''') Closer view of the boxed region shows colocalization of some *fezf2*-GFP+ cells with Hu (arrowhead). (D''') Inset shows colocalization in cells at higher magnification.

1.6 Summary

In this study, we have identified Fezf2 as a novel marker for radial glial progenitor cells of the adult zebrafish telencephalon (Figure 7, schematic). These Fezf2-expressing telencephalic ventricular progenitor cells label with markers of neural stem cells and proliferation. Furthermore, we have found *fezf2*-expressing progenitors and differentiated cell types in the olfactory bulb, the preoptic region, and the hypothalamus. Together with the previous studies in mice that have identified proliferating cells in the adult hypothalamus (Kokoeva et al., 2005; Kokoeva et al., 2007), our findings suggest that the hypothalamus represents another evolutionarily conserved region (in addition to the SVZ and hippocampus) that undergoes adult neurogenesis in the vertebrate brain, which is demarcated by *fezf2* expression.

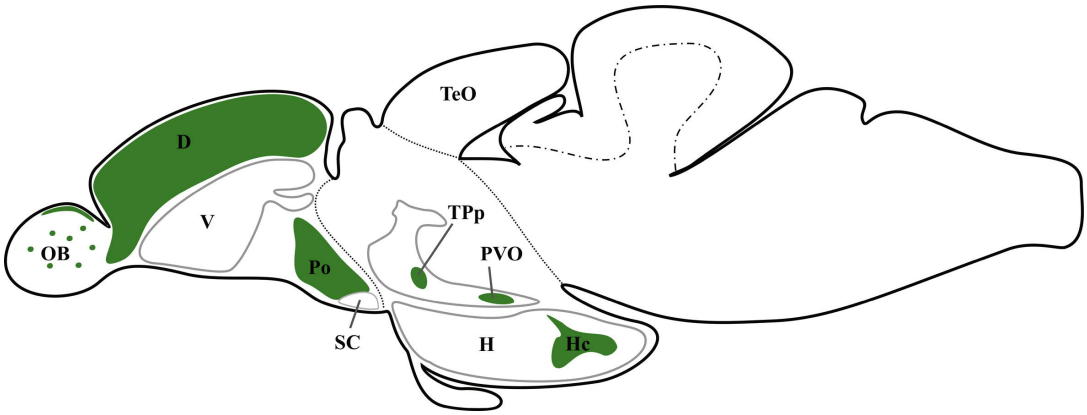


Figure 7. Schematic representation of *fezf2*-GFP expression in the adult zebrafish brain, lateral view. As described, *fezf2*-GFP expression is restricted to the forebrain and is detected in the olfactory bulb (OB), dorsal telencephalon (D), preoptic region (Po), posterior tuberculum (TPp, PVO), and caudal hypothalamus (Hc). *fezf2*-GFP+ cells are detected in the periphery of the caudal olfactory bulb. *fezf2* expression in the telencephalon is largely restricted to the pallial ventricular zone, where it marks radial glial progenitor cells. *fezf2*-GFP is expressed in all nuclei of the preoptic region, except SC. *fezf2*-GFP expression in the hypothalamus is localized exclusively to Hc (caudal hypothalamus). Schematic modified

from Wullimann et al., 1996. Abbreviations: **OB** olfactory bulb; **D** dorsal telencephalon; **V** ventral telencephalon; **Po** preoptic region; **SC** suprachiasmatic nucleus; **TPp** periventricular nucleus of posterior tuberculum; **PVO** paraventricular organ; **TeO** tectum opticum; **H** hypothalamus; **Hc** caudal hypothalamus.

The *fezf2*-GFP expressing differentiated cells in the adult zebrafish brain may represent adult-born neurons or glial cells derived from *fezf2*-expressing ventricular progenitor cells, which may or may not retain *fezf2* gene expression. Alternatively, *fezf2* may be expressed at low levels in these cells and is not detected by *in situ* hybridization; thus, these differentiated cells may be of embryonic origin that continue to express *fezf2*. It is also possible that these cells express GFP ectopically due to a lack of certain repressive elements in the *fezf2* promoter used to drive GFP. Future lineage tracing experiments are needed to help further discern some of these possibilities.

Taken together, our work lays a critical foundation for the use of Fezf2 as a novel marker to better understand the multipotent and self-renewal capacities of adult neural stem cells in zebrafish and sets the stage for the investigation of Fezf2 function in the maintenance and/or differentiation of neural stem cells in the adult vertebrate brain.

2. Experimental procedures

2.1 Zebrafish strains and maintenance

5- to 12-month old wild-type zebrafish (*Danio rerio*) of the AB strain and *fezf2*-GFP transgenic fish in the AB background were used. All procedures using animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

2.2 Generation of transgenic zebrafish

Transgenesis was carried out as previously described (Jeong et al., 2007). In brief, ~2.5 kb DNA fragment 5' and ~3 kb DNA fragment 3' to the *fezf2* gene were used to drive GFP reporter in a Tol2 transposon vector (Kawakami et al., 2004). The DNA construct was injected into early zebrafish embryos, which were raised to adulthood.

2.3 In situ hybridization

For *in situ* hybridization, cDNA encoding zebrafish *fezf2* was digested by SalI and transcribed using T7 RNA polymerase and a digoxigenin (DIG) labeling mix to generate antisense RNA probes. Adult zebrafish were anesthetized using tricaine and were perfused with phosphate buffer (PB) and 4% paraformaldehyde (Pfa). Fish were fixed in paraformaldehyde overnight and brains were dissected out the following day, embedded in gelatin-albumin, and processed for Vibratome sectioning and subsequent *in situ* hybridization procedure as previously described (Adolf et al., 2006).

2.4 BrdU labeling

Fish were anesthetized with Tricaine and injected intraperitoneally (I.P.) with a 10mM solution of Bromodeoxyuridine (BrdU) diluted in PBS twice with a 3-hour interval, followed by a survival time of 24 hours after the last injection. Fish were anesthetized, perfused with phosphate buffer (PB) and 4% paraformaldehyde (Pfa), and were fixed overnight. Brains were dissected out the following day, followed by cryoprotection overnight (30% sucrose in PB). Brains were embedded in OCT, and cryosectioning was performed (18 micron-thick). Immunohistochemistry was performed as described below (note modification to protocol for BrdU staining).

2.5 Immunohistochemistry and imaging

Adult zebrafish were processed as described above, followed by cryoprotection overnight (30% sucrose in PB). Brains were embedded in OCT, and cryosectioning was performed (18 micron-thick). Cryosections were washed in PBS (3X, 10 minutes), followed by washes in PBS + 0.5% Triton (2X, 5 minutes), and PBS (3X, 10 minutes). Cryosections were blocked in 3% BSA in PBS for 30 minutes and were incubated with primary antibodies (diluted in 3% BSA) overnight at 4 degrees C. The following day, sections were washed in PBS (3X, 10 minutes), followed by washes in PBS + 0.1% Triton (2X, 5 minutes). Sections were then incubated with secondary antibodies (diluted in PBS + 0.1% Triton) for 2 hours, followed by washes with PBS (6X, 10 minutes). Slides were then mounted (using Dako fluorescent mounting medium) and coverslipped. Modification to protocol: For BrdU staining, the sections were incubated in 4N HCl for 20 minutes at room temperature and washed with PBS (3X, 10 minutes) before washes

with PBS + 0.5% Triton. For PCNA staining, the sections were steamed for 5 minutes in citrate buffer and washed with PBS (3X, 10 minutes) before washes with PBS + 0.5% Triton. The following primary antibodies were used: anti-GFP (chicken, 1:2500, Abcam), anti-HuC/D (mouse, 1:1000, Invitrogen), anti-BLBP (rabbit, 1:400, Abcam), anti-GFAP (rabbit, 1:1000, Chemicon), anti-Sox3 (rabbit, 1:1000, a gift from Dr. M. Klymkowsky), anti-PCNA (mouse, 1:500, Dako), anti-BrdU (mouse, 1:200, Roche), anti-PH3 (rabbit, 1:2000, Upstate). The following secondary antibodies were used (Alexa, 1:200 dilution): anti-chicken 488, anti-mouse 568, anti-rabbit 568.

Images were obtained using a Leica confocal microscope, and Adobe Photoshop CS was used for image processing. Single optical Z-sections of ~0.5 micron were used when appropriate to assess colocalization of markers.

3. Acknowledgements

This work was supported by the NIH grant NS042626. We would like to thank Laure Bally-Cuif for the *in situ* hybridization protocol, as well as M. Klymkowsky who provided the anti-Sox3 antibody as a kind gift. We would also like to thank laboratory members for helpful discussions.

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CHAPTER 2

fezf2 regulates the proliferation and differentiation of neural progenitor cells in the adult zebrafish telencephalon

Abstract

Neural stem cells of the mammalian subventricular zone (SVZ) and hippocampal subgranular zone (SGZ) continuously give rise to new neurons in the adult brain. Though some progress has been made in understanding the role of cell intrinsic factors in the regulation of adult neural stem cells, our knowledge remains limited. We have recently reported that *fezf2*, a conserved forebrain-specific zinc finger transcription factor, is expressed in radial glial-like cells of the adult zebrafish telencephalic ventricular zone (VZ), which label with markers of neural stem cells (NSCs) and proliferation. Through BrdU and EdU pulse-chase studies in *fezf2*-GFP transgenic zebrafish, we now show that these *fezf2*-GFP+ cells have the ability to self-renew and may be able to give rise to new glutamatergic neurons of the Dm region in the adult zebrafish telencephalon. Analysis of *too few* (Fezf2) homozygous mutant zebrafish reveals a previously unreported adult telencephalic phenotype, characterized by a somewhat malformed and overall smaller-sized telencephalon, while the rest of the brain appears normal. By crossing our *fezf2*-GFP reporter line to the Fezf2 mutant background, we find an increase in the proliferation of *fezf2*-GFP+ cells of the adult mutant telencephalic VZ. We also find an overall increase in the number of neurons near *fezf2*-GFP+ cells of the adult mutant

telencephalic VZ, and an increase in adult telencephalic neurogenesis, suggesting that Fezf2 functions normally to maintain neural progenitor cells in a non-proliferative, or inactive, state. No major differences are observed in the telencephalon of larval mutant zebrafish (14dpf), suggesting that the mutant phenotype occurs largely at the late-larval to adult stage. Moreover, results obtained from the transplantation of *fezf2*-GFP+ mutant cells into wildtype hosts suggest that Fezf2 functions cell-autonomously to regulate the proliferation of telencephalic radial glial-like cells. Taken together, our findings demonstrate a role for Fezf2 in maintaining the non-proliferative state of neural progenitor cells in the adult vertebrate telencephalon.

Introduction

The birth of new neurons in the adult brain, termed adult neurogenesis, is a process that occurs continuously and robustly from neural stem cells (NSCs) located in two regions of the adult mammalian brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus (Alvarez-Buylla and Garcia-Verdugo, 2002; Doetsch et al., 1999; Gage et al., 2000; Garcia et al., 2004; Goldman, 2003; Merkle et al., 2004; Taupin and Gage, 2002). Adult neural stem cells of the SVZ give rise to new neurons of the olfactory bulb, while neural progenitor cells of the SGZ give rise to neurons that remain within the hippocampus. Some progress has been made in understanding the role of cell intrinsic factors (i.e. transcription factors) in the regulation of adult NSCs (Zhao et al., 2008; Episkopou, 2005; Ferri et al., 2004). However, our understanding of the factors that regulate the maintenance and/or differentiation of adult NSCs remains limited.

Fezf2, a conserved forebrain-specific zinc finger transcription factor (Shimizu and Hibi, 2009), is expressed during development in both zebrafish and mouse (Hashimoto et al., 2000; Hirata et al., 2004; Inoue et al., 2004; Jeong et al., 2007). Previous studies in both systems indicate a conserved role for Fezf2 in patterning of the diencephalon (Hirata et al., 2006; Jeong et al., 2007). Studies in zebrafish using the *too few* mutant, which was identified in a screen for an almost complete loss of hypothalamic dopamine neurons (Guo et al., 1999) and was later found to contain a mutation in the *fezf2* gene (Levkowitz et al., 2003), have suggested a role for *fezf2* in embryonic neurogenesis. Additionally, analysis of the adult *too few* (Fezf2) mutant hypothalamus has revealed deficits in neuronal number at the adult stage (Rink et al., 2004).

In mouse, Fezf2 is required for the formation of corticospinal motor neurons of cortical layer 5 (Chen et al., 2005a, 2005b; Molyneaux et al., 2005), and recent reports indicate additional roles for Fezf2 in neuronal cell type specification (Chen et al., 2008) and in the directed differentiation of neurons of the glutamatergic lineage (Rouaux and Arlotta, 2010). Additionally, some progress has been made in understanding the role of Fezf2 in neuronal differentiation from neural progenitor cells in the developing mouse forebrain (Shimizu et al., 2010), and a role for this gene in regulating neural progenitor cells during invertebrate development as well (Weng et al., 2010). However, Fezf2 function in adult neural stem cells and adult neurogenesis has not previously been investigated.

We have recently reported that *fezf2* is expressed in radial glial-like cells of the adult zebrafish telencephalic ventricular zone (VZ), which label with markers of neural stem cells and proliferation (Berberoglu et al., 2009). Building on these findings, we again use the zebrafish, which has recently emerged as a new vertebrate system to study adult neurogenesis (Adolf et al., 2006; Bae et al., 2009; Byrd and Brunjes, 1998; Chapouton et al., 2006; Chapouton et al., 2007; Chapouton and Godinho, 2010; Grandel et al., 2006; Kani et al., 2010; Kaslin et al., 2009; Lam et al., 2009; Zupanc, 2008; Zupanc et al., 2005), to investigate the functional role of *fezf2* in adult neural stem cells and adult neurogenesis.

This required us to first establish that *fezf2*-expressing radial glial-like cells do in fact act as neural stem cells in the adult zebrafish telencephalon by determining whether they are able to self-renew and give rise to differentiated cell types. To this aim, we administered a pulse of BrdU, followed by a 2-week chase period, and found *fezf2*-GFP+

label-retaining cells, some of which were also positive for EdU, which was administered at the end of the BrdU chase period. This suggests that *fezf2*-GFP+ cells can go through multiple rounds of divisions and provides evidence for their self-renewal. Through BrdU pulse-chase studies, we also show that *fezf2*-GFP+ cells may be able to give rise to *vglut2a*-GFP+ glutamatergic neurons of the Dm region in the adult zebrafish telencephalon. These findings demonstrate that *fezf2*-GFP+ cells have neural stem cell-like properties, and may thus represent neural stem cells in the adult zebrafish telencephalon.

To determine whether Fezf2 plays a functional role in the proliferation or differentiation of adult neural progenitor cells, we performed analysis on the adult *too few* (Fezf2) mutant brain. In analyzing the adult *too few* mutant brain, we find a striking and specific telencephalic phenotype, with homozygous mutant telencephalon being malformed and overall smaller in size. By crossing the *too few* mutant to our *fezf2*-GFP reporter line, analysis on sections reveals an increase in proliferation of *fezf2*-GFP+ cells, an overall increase in neurons, and an increase in neurogenesis in the adult mutant telencephalon. No major differences are observed in the mutant telencephalon at the larval stage (14dpf), suggesting that the mutant phenotype occurs mostly at the late-larval to adult stage.

To determine whether Fezf2 functions autonomously in telencephalic radial glial-like cells to regulate their proliferation, *fezf2*-GFP+ mutant cells were transplanted into wildtype hosts, and hosts were grown to the adult stage for analysis. My preliminary data show that *fezf2*-GFP+ transplanted heterozygous cells proliferate more than neighboring

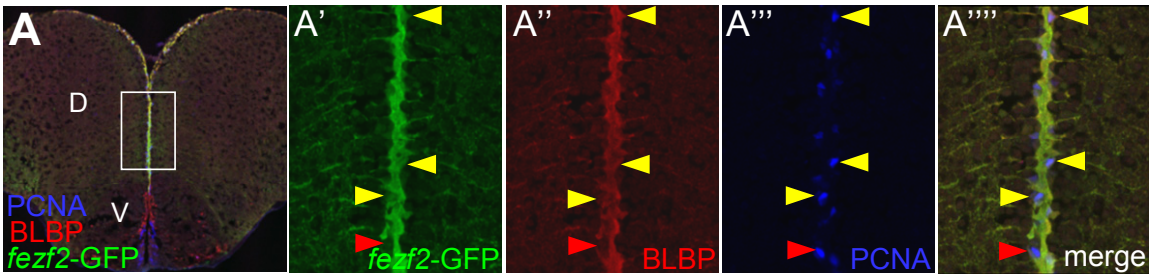
untransplanted cells, supporting a cell-autonomous role for *fezf2* in regulating the proliferation of *fezf2*-GFP+ neural progenitor cells.

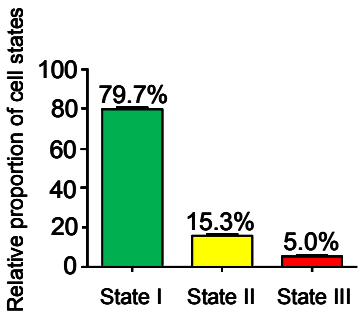
Taken together, our findings suggest a role for *fezf2* in maintaining the non-proliferative state of neural progenitor cells within the adult zebrafish telencephalon.

Results

Categorization of fezf2-GFP+ cells among progenitor cells within the adult zebrafish telencephalic ventricular zone

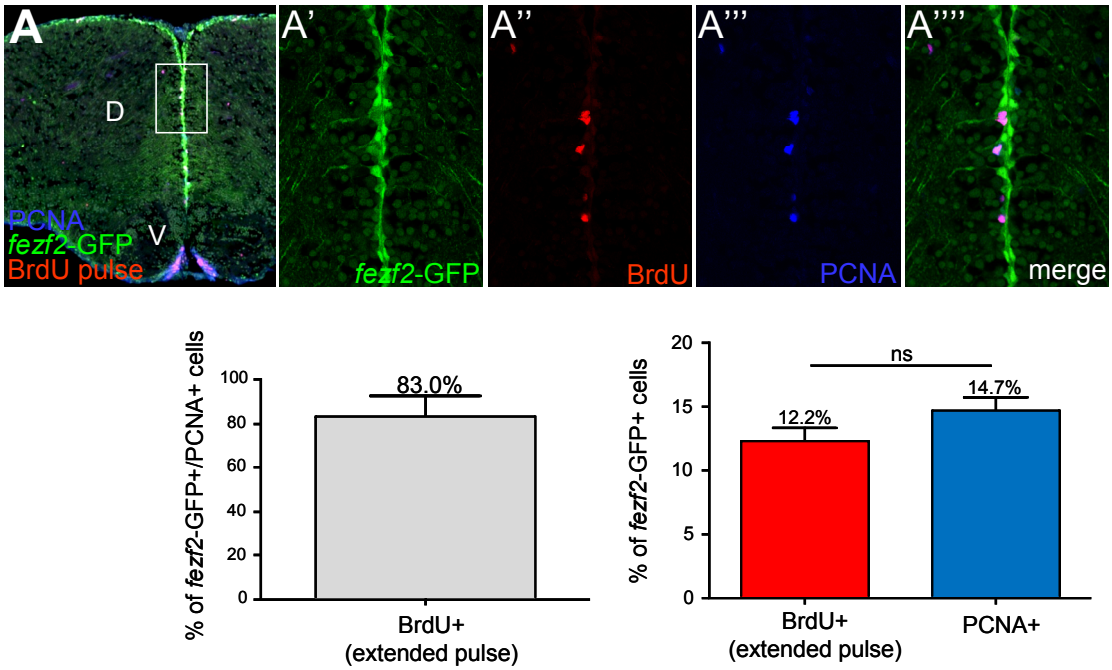
Recent studies establishing the existence of diverse progenitor cell states in the adult zebrafish telencephalic VZ (Chapouton et al., 2010; Ganz et al., 2010; März et al., 2010) have allowed us to classify our *fezf2*-GFP+ cells among the State I (non-proliferating radial glial-like cells), State II (proliferating radial glial-like cells), and State III (proliferating non-radial glial) progenitor cells. We find that *fezf2*-GFP+ cells represent a population of largely non-proliferating radial glial-like cells, with ~80% of telencephalic VZ cells being State I *fezf2*-GFP+ cells that are PCNA-negative (marker of actively cycling cells). Proliferating *fezf2*-GFP+ State II cells make up a small fraction (~15%) of the total population of VZ cells, and ~5% of VZ cells are *fezf2*-GFP-negative non-radial glial proliferating cells (State III cells) (n=3 brains; **Supplemental Fig. S1**). We also noticed that proliferating *fezf2*-GFP+ cells have weaker GFP expression than non-proliferating *fezf2*-GFP+ cells, an observation that we are interested in following up on in future experiments.





Supplementary Figure S1. Categorization of *fezf2*-GFP+ cells among State I, State II, and State III progenitor cells within the adult zebrafish telencephalic ventricular zone.
(A) Coronal section through adult zebrafish telencephalon with triple-labeling for *fezf2*-GFP, PCNA (marker of proliferation), and BLBP (marker of neural stem cells) (20X magnification; confocal projection image). (A'-A'''') 100X magnification (single confocal z-section) of boxed region in (A) showing overlap of *fezf2*-GFP and BLBP, as well as colocalization of *fezf2*-GFP and PCNA. State II (*fezf2*-GFP+/PCNA+) cells are depicted by yellow arrowheads, while State III (*fezf2*-GFP-/PCNA+) cells are depicted by red arrowhead. State I (*fezf2*-GFP+/PCNA-) cells are present but are not depicted by arrowheads. Relative proportion of cells states is quantified (n=3 brains). Abbreviations: D dorsal telencephalon, V ventral telencephalon.

Validation of PCNA as a reliable marker for proliferation was performed by administering an extended pulse (see Materials & Methods) of BrdU, followed by triple-labeling for *fezf2*-GFP/PCNA/BrdU. A majority (83.0%) of *fezf2*-GFP+/PCNA+ cells are BrdU+ following an extended pulse of BrdU (n=3 brains; **Supplemental Fig. S2**), suggesting that PCNA is a reliable marker for actively cycling cells.



Supplemental Figure S2. Validation of PCNA as a reliable marker for proliferating cells. (A) Coronal section through adult *fezf2*-GFP+ telencephalon with triple-labeling for *fezf2*-GFP/BrdU/PCNA following an extended pulse of BrdU (3 I.P. injections per day for a period of 2 days) (20X magnification, confocal projection image). (A'-A'''') 100X magnification (single confocal z-section) of boxed region in (A) showing that most *fezf2*-GFP+/PCNA+ cells are also BrdU+ (marker of S-phase) following an extended pulse of BrdU, indicating that PCNA accurately labels cells that are actively cycling. (B) Quantification indicates that 83.0% of *fezf2*-GFP+/PCNA+ cells are also BrdU+ after an extended pulse of BrdU. (C) No significant differences are observed in the number of *fezf2*-GFP+ cells that are BrdU+ (12.2%) and the number of *fezf2*-GFP+ cells that are PCNA+ (14.7%) (n=3 brains). Abbreviations: D dorsal telencephalon, V ventral telencephalon.

BrdU and EdU labeling experiments indicate that fezf2-GFP+ cells can self-renew in vivo

To further determine whether these *fezf2*-GFP+ radial glial-like cells are acting as neural stem cells in the adult zebrafish telencephalon, we performed BrdU/EdU pulse-chase experiments in *fezf2*-GFP transgenic zebrafish to determine whether these *fezf2*-GFP+ cells have the ability to self-renew. An extended pulse of BrdU (schematized in **Fig. 1A**) was administered I.P., followed by a 2-week chase period and ending with a pulse of EdU. *fezf2*-GFP transgenic zebrafish were sacrificed 24 hours after the EdU pulse, and brains were processed for immunostaining. The presence of *fezf2*-GFP+/BrdU+/EdU+ triple-labeled cells suggests that *fezf2*-GFP+ cells have the ability to self-renew, as they go into S-phase once during the BrdU pulse and again during the EdU pulse, suggesting that these cells are going through multiple rounds of self-renewing divisions (**Fig. 1B**). As only 0.6% of *fezf2*-GFP+ cells are BrdU and EdU-positive (n=3 brains; **Fig. 1D**), these results also suggest that *fezf2*-GFP+ cells are relatively inactive as a population, which is characteristic of adult neural stem cells.

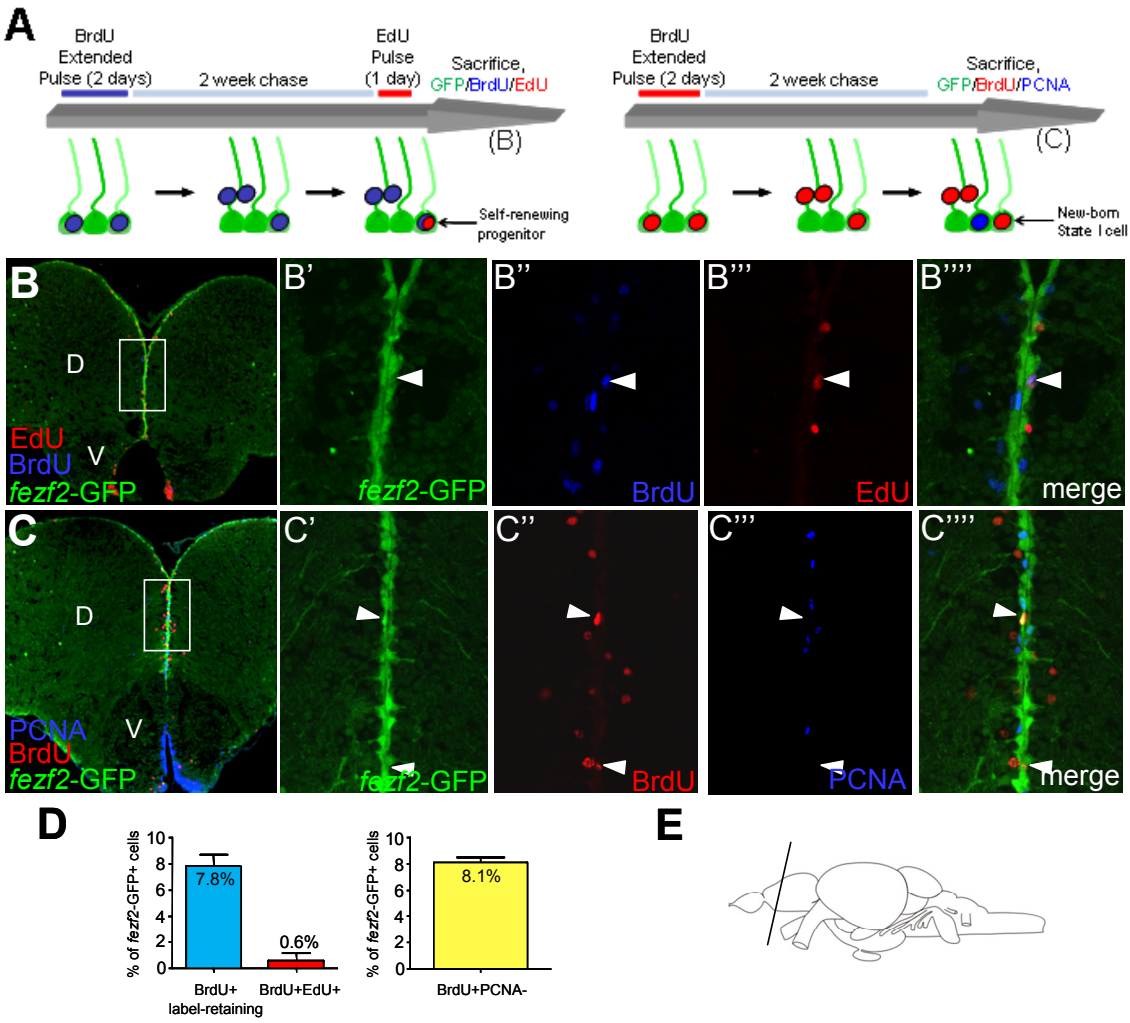
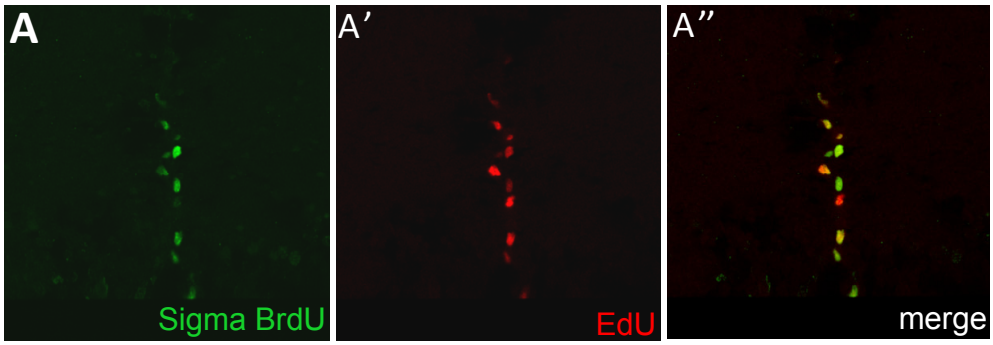


Figure 1. *fezf2*-GFP+ radial glial cells of the adult zebrafish telencephalic ventricular zone can self-renew *in vivo*.

(A) Schematic diagram describing experimental design used to determine whether *fezf2*-GFP+ cells can self-renew (experimental data shown in (B)). Schematic diagram describing experimental design used to determine whether new State I cells are born (experimental data shown in (C)). (B) *fezf2*-GFP+ cells can self-renew *in vivo*. Triple-labeling of *fezf2*-GFP/BrdU/EdU (20X magnification; confocal projection image). (B'-B'''') 100X magnification (single confocal z-section) of boxed region in (B) showing the presence of triple-labeled cells, indicated by arrowhead. *Fezf2*-GFP+/BrdU+ label-retaining cells and *fezf2*-GFP+/BrdU+/EdU+ triple-labeled cells are quantified in (D). (C) *fezf2*-GFP+ State II (proliferating) cells can give rise to State I (non-proliferating) cells, indicating that new *fezf2*-GFP+ State I cells are born. Triple-labeling of *fezf2*-GFP/BrdU/PCNA (20X magnification; confocal projection image). (C'-C'''') 100X magnification (single confocal z-section) of boxed region in (C) showing *fezf2*-GFP+/BrdU+/PCNA- cells (indicated by arrowheads), suggesting that new State I cells are born. The percentage of *fezf2*-GFP+ cells that are

BrdU+ and PCNA- is quantified in (D). (D) Quantification of data shown in (B) and (C) (n=3 brains for each condition). (E) Schematic drawing showing lateral view of adult zebrafish brain, with plane of section through telencephalon indicated. Abbreviations: D dorsal telencephalon, V ventral telencephalon.

BrdU and EdU double-labeling procedure was optimized using a Sigma anti-BrdU antibody which does not cross-react with EdU. As shown in **Supplemental Fig. S3**, the same cells are labeled with BrdU and EdU, suggesting that EdU is a reliable marker for cells in S-phase.

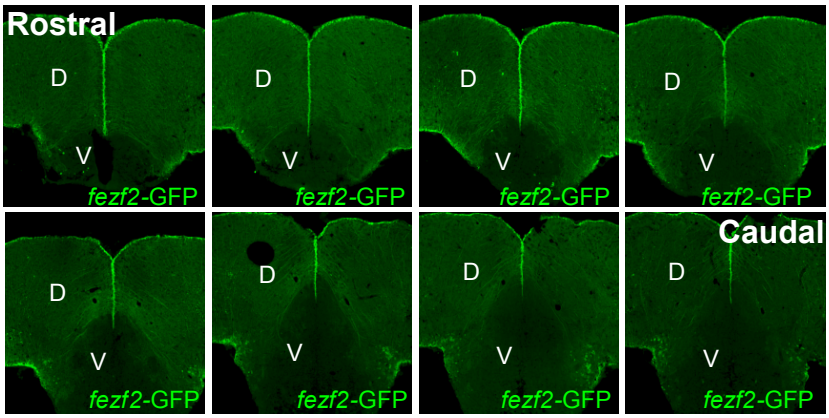


Supplemental Figure S3. Optimization of BrdU and EdU double-labeling within the adult zebrafish telencephalon.
(A-A'') Single injections of BrdU and EdU were administered I.P. (intraperitoneal) in separate injections (~15-20 minutes apart). EdU click chemistry reaction was performed, followed by antibody labeling with a Sigma anti-BrdU antibody (which does not cross react with EdU). Note that BrdU and EdU are labeling the same cells but at different intensities, which may be due to the time between BrdU and EdU injections, or may be due to different sensitivity.

To determine whether *fezf2*-GFP+ State I (non-proliferating) and State II (proliferating) cells represent two distinct populations of cells, or whether State II cells are able to give rise to State I cells, we administered an extended pulse of BrdU, followed by a 2-week chase period, and subsequent staining for *fezf2*-GFP/BrdU/PCNA (schematized in **Fig 1A**). The presence of *fezf2*-GFP+/BrdU+/PCNA- cells (8.1% of total

fezf2-GFP+ population) indicates that *fezf2*-GFP+ State II cells that are proliferating and go into S-phase during the BrdU pulse are able to give rise to *fezf2*-GFP+ State I cells that are no longer cycling (n=3 brains; **Fig. 1C, D**). These data suggest that *fezf2*-GFP+ cells represent a population of radial glial-like cells that can transit back and forth between State I (non-proliferating) and State II (proliferating) progenitor cells and are able to self-renew and maintain themselves in this way.

Serial sections showing the rostro-caudal extent of *fezf2*-GFP expression through the telencephalon are provided (see **Supplemental Fig. S4**).



Supplemental Figure S4. Serial sections through telencephalon of adult *fezf2*-GFP transgenic line shows rostro-caudal extent of *fezf2*-GFP expression (20X confocal projection images).
Abbreviations: D dorsal telencephalon, V ventral telencephalon.

Vglut2a-GFP+ glutamatergic neurons are produced from ventricular progenitor cells within the Dm region of the telencephalon

fezf2-GFP+ radial glial-like cells reside in the medial part of the dorsal telencephalon (Dm region). Adjacent to these *fezf2*-GFP+ cells are stripes of HuC/D+ neurons, as we and others have previously shown (Adolf et al., 2006; Berberoglu et al., 2009; Grandel et al., 2006). Using a *vGlut2a*-GFP transgenic zebrafish line, we now show that a vast majority (74.7%) of these pallial HuC/D+ neurons are glutamatergic *vGlut2a*-GFP+ cells, providing a specific neurotransmitter identity to these HuC/D+ neurons of the zebrafish dorsal telencephalon (n=3 brains; **Fig. 2A, B**).

By performing BrdU pulse-chase studies, we show that *vGlut2a*-GFP+ cells are continuously being produced in the adult zebrafish Dm region. 20.6% of *vGlut2a*-GFP+ cells were BrdU+ following an extended pulse (3 injections per day for 2 days), and followed by a 2-week chase period (n=3 brains; **Fig. 2F, G**). Furthermore, our data suggest that these *vGlut2a*-GFP+ new neurons may be born from *fezf2*-GFP+ neural progenitor cells, as an extended pulse of BrdU (without chase period) labels a number of *fezf2*-GFP+/BLBP+ progenitor cells but no *vGlut2a*-GFP+ cells (n=3 brains for each condition; **Fig. 2C-E**).

In situ hybridization for *vglut2a* on serial vibratome sections (100uM) through the adult zebrafish telencephalon confirms mRNA expression in a pattern similar to that of the *vglut2a*-GFP transgenic line (**Supplemental Fig. S5**). Serial sections through *vglut2a*-GFP telencephalon are also provided in **Supplemental Fig. S5**.

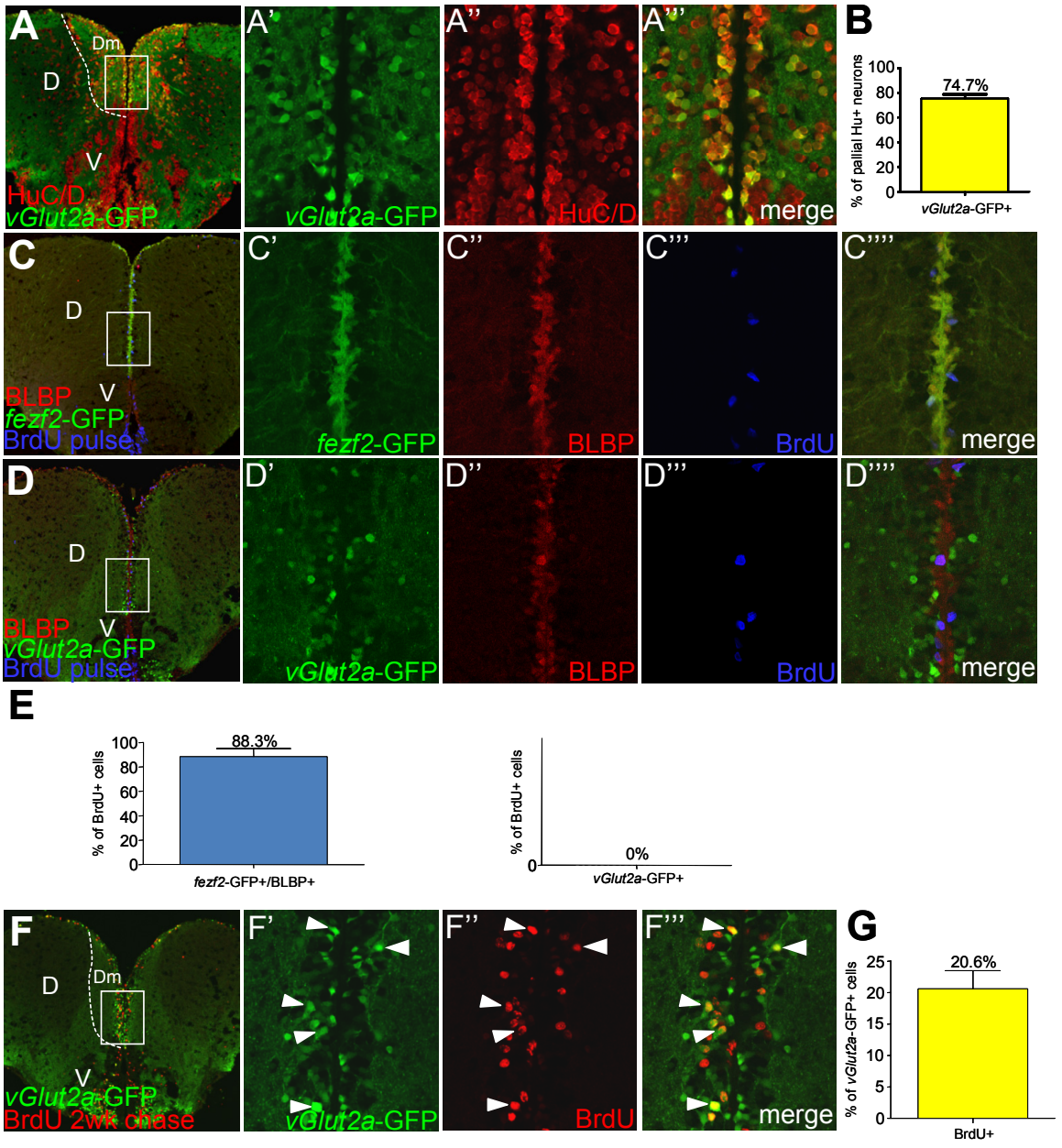
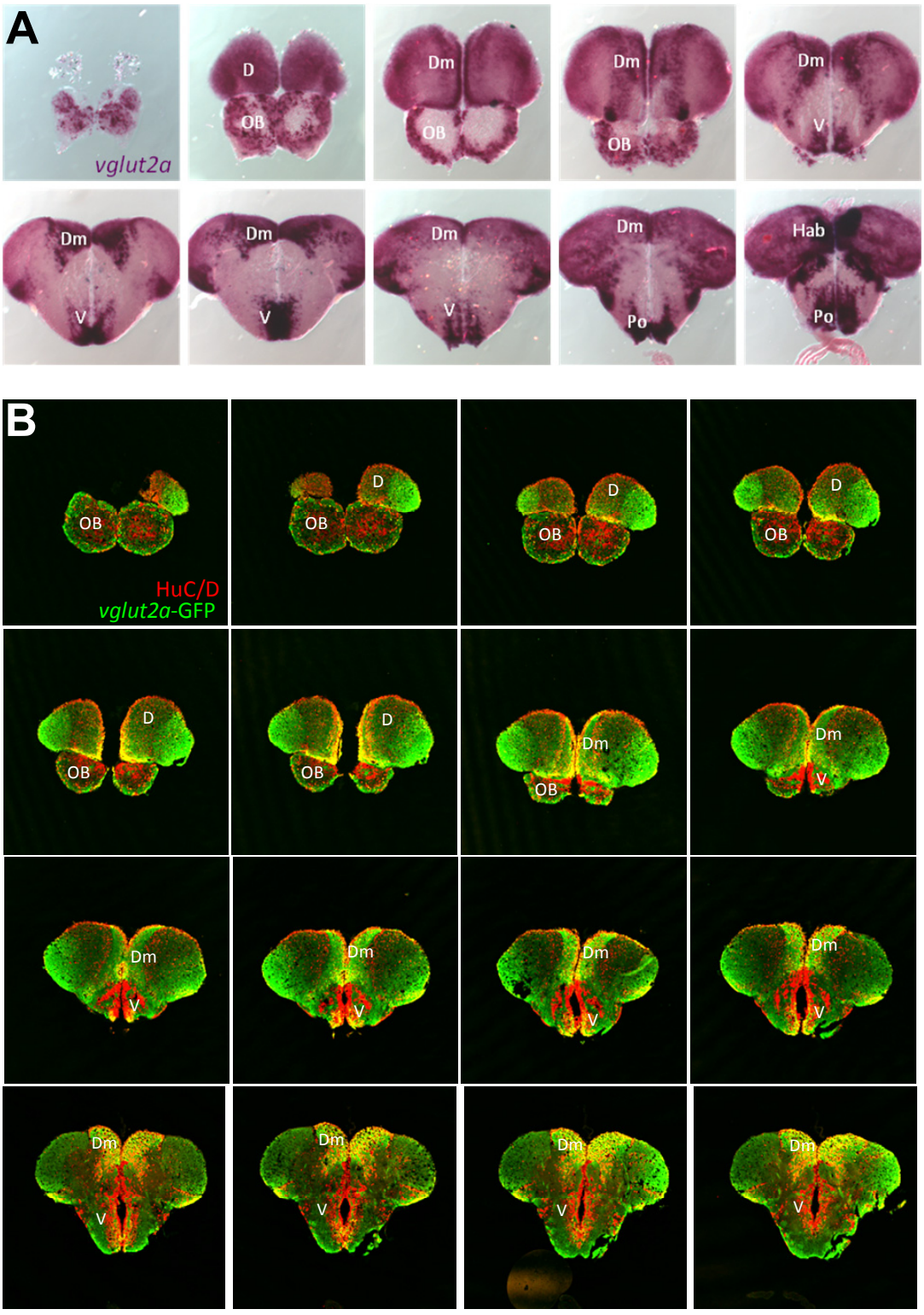


Figure 2. *vglut2a*-GFP+ glutamatergic neurons are produced from ventricular progenitor cells within the Dm region of the adult zebrafish telencephalon. (A) Identification of a majority of neurons within the medial part of the dorsal telencephalon (Dm) as *vglut2a*-GFP+ glutamatergic cells. Coronal section through telencephalon of *vglut2a*-GFP transgenic brain together with HuC/D (neuronal marker) labeling (20X magnification; confocal projection image). Dm region is indicated by dotted line. (A'-A''') 100X magnification (single confocal z-section) of boxed region in (A) shows that a majority of HuC/D+ neurons in this region are *vglut2a*-GFP+. (B) Quantification indicates that 74.7% of HuC/D+ neurons in this region are *vglut2a*-GFP+ (n=3 brains). (C) Acute BrdU labeling in *fezf2*-GFP transgenic line, with triple-labeling for *fezf2*-GFP/BrdU/BLBP (20X

magnification; confocal projection image). (C'-C'''') 100X magnification (single confocal z-section) of boxed region in (C) shows that a pulse of BrdU (2 injections) labels cells in the ventricular zone, a majority of which are *fezf2*-GFP+ as quantified in (E). (D) Acute BrdU labeling in *vglut2a*-GFP transgenic line, with triple labeling for *vglut2a*-GFP/BrdU/BLBP (20X magnification; confocal projection image). (D'-D'''') 100X magnification (single confocal z-section) of boxed region in (D) shows that a pulse of BrdU (2 injections) labels cells in the ventricular zone, but no *vglut2a*-GFP+ cells (quantified in E). (E) Quantification indicates that 88.3% of BrdU+ cells are *fezf2*-GFP+/BLBP+ after an extended pulse of BrdU (3 injections per day for 2 days) (n=3 brains), and that 0% of BrdU+ cells are *vglut2a*-GFP+ after an extended pulse of BrdU (n=3 brains). (F) BrdU pulse-chase labeling in *vglut2a*-GFP transgenic line indicates that new *vglut2a*-GFP+ cells are born in the adult telencephalon following an extended pulse of BrdU and 2-week chase period. (F'-F''') 100X magnification (single confocal z-section) shows a number of *vglut2a*-GFP+/BrdU+ cells, indicating that these cells are continuously produced in the adult brain. (G) Quantification shows that 20.6% of *vglut2a*-GFP+ cells are BrdU+ following an extended pulse and 2-week chase period (n=3 brains). Abbreviations: D dorsal telencephalon, V ventral telencephalon, Dm (medial part of the dorsal telencephalon).



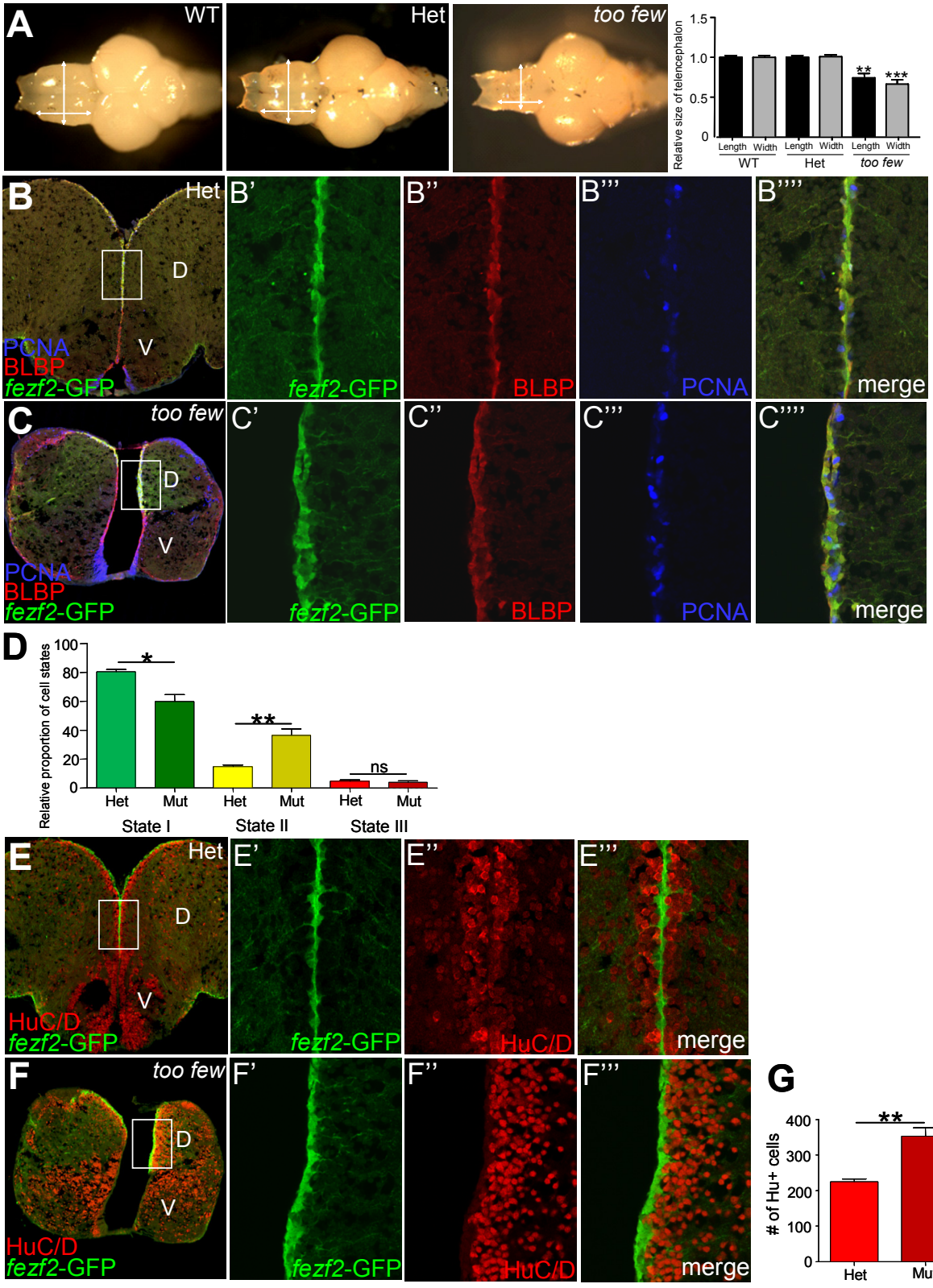
Supplemental Figure S5. *In situ* hybridization for *vglut2a* mRNA on 100uM serial vibratome sections through adult zebrafish telencephalon, and serial sections through telencephalon of *vglut2a*-GFP transgenic line, double-labeled with HuC/D (neuronal marker).

(A) Note stripes of *vglut2a* mRNA expression near midline ventricular zone of the dorsal telencephalon, as well as strong expression throughout the Dm region. (B) *Vglut2a*-GFP expression colocalizes with HuC/D near the midline ventricular zone of the dorsal telencephalon, as well as throughout the Dm region, which is consistent with *vglut2a* mRNA expression and suggests that these cells are glutamatergic neurons. Abbreviations: OB olfactory bulb, D dorsal telencephalon, Dm medial part of the dorsal telencephalon, V ventral telencephalon, Po preoptic region, Hab habenula.

Taken together, these data suggest that *fezf2*-GFP+ radial glial-like cells may be acting as neural stem cells in the adult zebrafish telencephalon, retaining the ability to self-renew and also to give rise to differentiated neuronal cell types.

Adult too few (Fezf2) homozygous mutant telencephalon is smaller in size, and analysis reveals an increase in proliferation of fezf2-GFP+ cells and an increase in adult neurogenesis

Given *fezf2* expression in cells with neural stem cells-like properties, we went on to investigate the functional role of Fezf2 in the proliferation and differentiation of these cells. By analyzing the *too few* (Fezf2) mutant, we found that the adult mutant telencephalon is malformed and smaller in size compared to the telencephalon of wildtype or heterozygote siblings, while the rest of the brain appears normal (n=6 brains for each genotype; $p<0.01$ for length, $p<0.001$ for width; **Fig. 3A**).


A
WT
Het
too few
Relative size of telencephalon
1.5
1.0
0.5
0
**
***
Length
Width
Length
Width
Length
Width
WT
Het
too few
B
Het
D
V
PCNA
BLBP
fezf2-GFP
B'
fezf2-GFP
B''
BLBP
B'''
PCNA
B''''
merge
C
too few
D
V
PCNA
BLBP
fezf2-GFP
C'
fezf2-GFP
C''
BLBP
C'''
PCNA
C''''
merge
D
Relative proportion of cell states
100
80
60
40
20
0
*
**
ns
Het
Mut
Het
Mut
Het
Mut
State I
State II
State III
E
Het
D
V
HuC/D
fezf2-GFP
E'
fezf2-GFP
E''
HuC/D
E'''
merge
F
too few
D
V
HuC/D
fezf2-GFP
F'
fezf2-GFP
F''
HuC/D
F'''
merge
G
# of Hu+ cells
400
300
200
100
0
**
Het
Mut

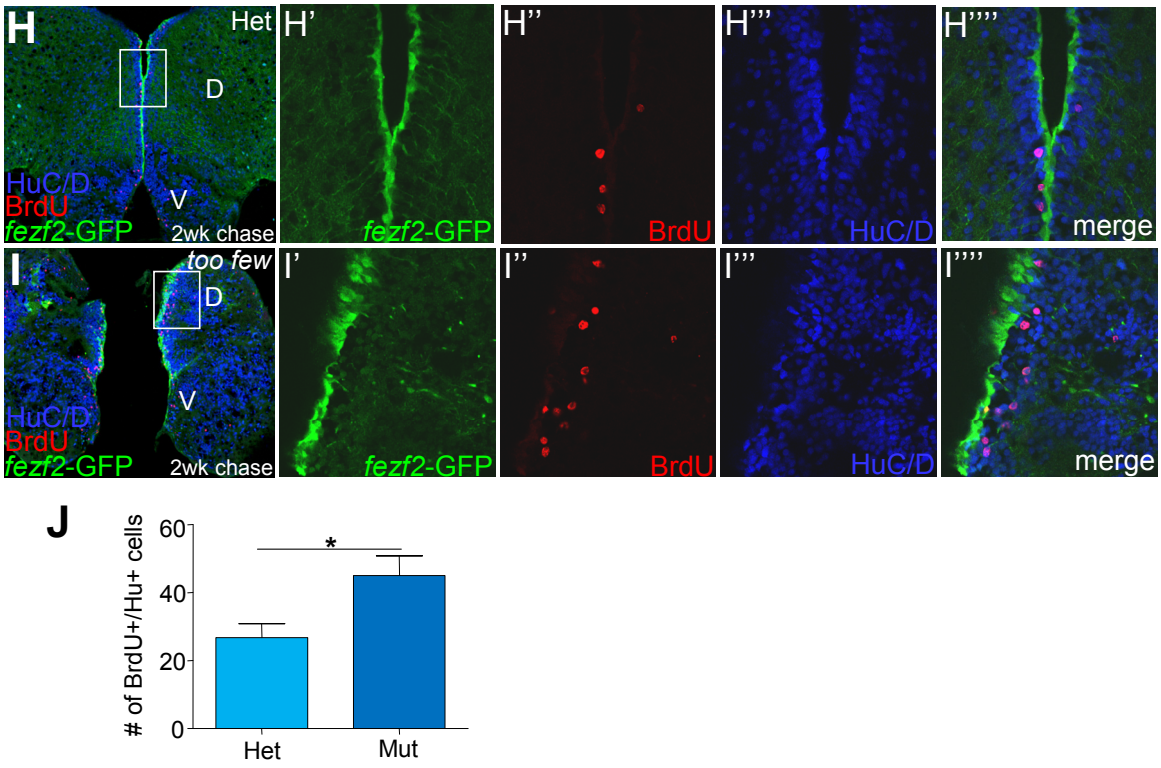


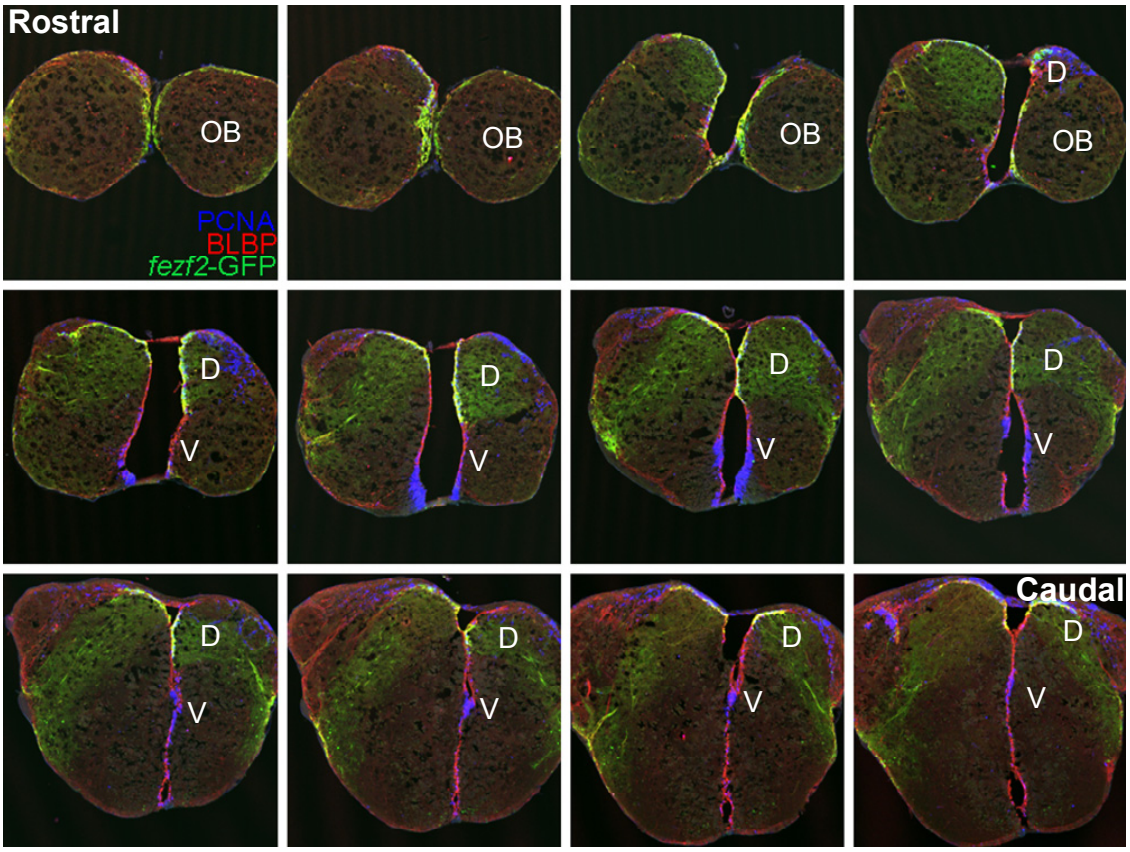
Figure 3. Adult *too few* (Fezf2) homozygous mutant telencephalon is substantially smaller in size, and analysis reveals an increase in proliferation of *fezf2*-GFP+ cells and an increase in adult neurogenesis.

(A) Dorsal view of adult wildtype, *too few* heterozygote, and homozygote brains shows that the *too few* homozygous mutant telencephalon is substantially smaller in size compared to wildtype and heterozygote siblings. Quantification of the relative size of the telencephalon compared to the rest of the brain indicates that this is significant (n=6 brains for each genotype; $p<0.01$ for length, $p<0.001$ for width, *t*-test). (B) Coronal section through *too few* heterozygote telencephalon, triple-labeled for *fezf2*-GFP/BLBP/PCNA (20X magnification, confocal projection image). (B'-B'''') 100X magnification (single confocal z-section) of boxed region in (B) shows overlap of *fezf2*-GFP and BLBP, as well as colocalization with PCNA (n=3 heterozygote brains). (C) Coronal section through *too few* homozygous mutant telencephalon, triple-labeled for *fezf2*-GFP/BLBP/PCNA (20X magnification, confocal projection image). (C'-C'''') 100X magnification (single confocal z-section) of boxed region in (C) shows an increase in proliferation of *fezf2*-GFP+ cells (n=3 homozygote brains). (D) Quantification reveals an increase in *fezf2*-GFP+/PCNA+ (State II) cells and a decrease in *fezf2*-GFP+/PCNA- (State I) cells in the homozygous mutant telencephalon compared to heterozygote sibling controls (n=3 heterozygote brains and 3 homozygote brains; $p<0.01$ for State II cells, $p<0.05$ for State I cells, *t*-test), suggesting an increase in proliferation of *fezf2*-GFP+ cells in the adult *too few* homozygous mutant telencephalon. (E) Coronal section through *too few* heterozygote telencephalon, double-labeled for *fezf2*-GFP and HuC/D

(neuronal marker) (20X magnification, confocal projection image). (E'-E''') 100X magnification (single confocal z-section) of boxed region in (E) showing neuronal composition near *fezf2*-GFP+ cells of the telencephalic VZ (n=3 heterozygote brains). (F) Coronal section through *too few* homozygous mutant telencephalon, double-labeled for *fezf2*-GFP and HuC/D (20X magnification, confocal projection image). (F'-F''') 100X magnification (single confocal z-section) of boxed region in (F) showing an increase in HuC/D+ neurons adjacent to *fezf2*-GFP+ cells of the telencephalic VZ (n=3 homozygote brains). (G) Quantification of HuC/D+ neurons near *fezf2*-GFP+ cells of the telencephalic VZ reveals a significant increase in the homozygous mutant telencephalon (n=3 heterozygote brains and 3 homozygote brains; $p<0.01$, *t*-test). (H-I) BrdU pulse-chase experiments in which an extended pulse of BrdU (3 injections per day for 2 days) was administered, followed by a 2-week chase period. (H) Triple-labeling for *fezf2*-GFP/BrdU/Hu in *too few* heterozygote telencephalon after 2-week chase period (20X magnification, confocal projection image). (H'-H'''') 100X magnification (single confocal z-section) of boxed region in (H) indicates that new neurons are born within 2-week chase period (n=3 heterozygote brains). (I) Triple-labeling for *fezf2*-GFP/BrdU/Hu in *too few* homozygous mutant telencephalon after 2-week chase period (20X magnification, confocal projection image). (I'-I'''') 100X magnification (single confocal z-section) of boxed region in (I) shows an increase in adult neurogenesis in homozygous mutant telencephalon with a 2-week chase period (n=3 homozygote brains). (J) Quantification of adult-born (BrdU+/Hu+) neurons in *too few* heterozygous and homozygous mutant telencephalon indicate that there is a significant increase in adult neurogenesis in homozygous mutant telencephalon (n=3 heterozygote and 3 homozygote brains; $p<0.05$, *t*-test). *$p<0.05$; **$p<0.01$; ***$p<0.001$. Abbreviations: D dorsal telencephalon, V ventral telencephalon.

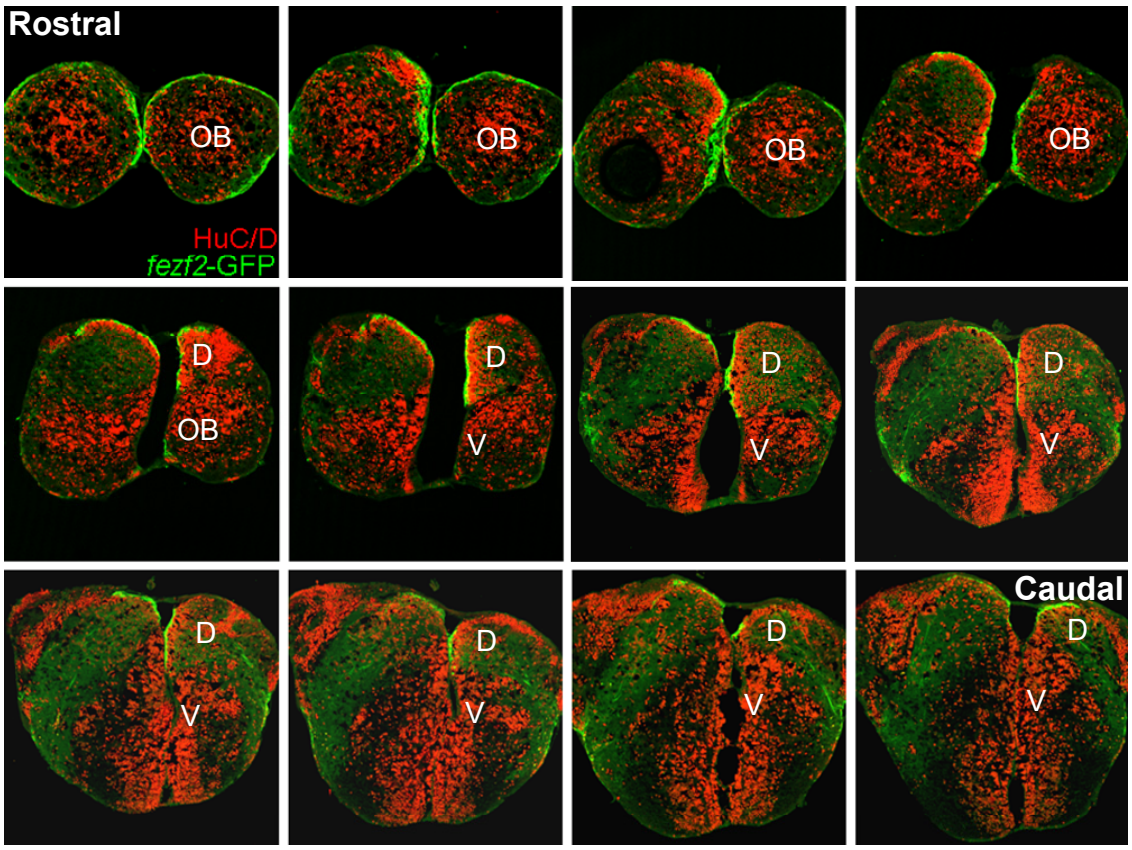
Analysis of Fezf2 mutants crossed to our *fezf2*-GFP reporter line reveals that the dorsal telencephalon (D; *fezf2*-GFP-positive domain) is significantly smaller in size, and *fezf2*-GFP+ progenitor domains are often shifted, which appears to be due to an expansion of the ventral telencephalon (V; *fezf2*-GFP-negative domain) (**Fig. 3B, C**). Most notably, we find an increase in the proliferation of *fezf2*-GFP+ cells of the dorsal telencephalic ventricular zone (indicated by PCNA labeling) compared with heterozygote siblings (**Fig. 3B-B'''', C-C''''**). Quantification reveals an increase in the proportion of State II (proliferating) *fezf2*-GFP+ cells at the expense of State I (non-proliferating) *fezf2*-GFP+

cells (n=3 heterozygote and 3 homozygote brains; $p<0.01$ for State II cells, $p<0.05$ for State I cells; **Fig. 3D**). Though it is possible that the phenotype we are observing is due, in part, to compaction of the dorsal telencephalon, an increase in the relative proportion of proliferating versus non-proliferating *fezf2*-GFP+ cells suggests that the phenotype is not simply due to compaction. Serial sections through adult mutant telencephalon are provided to better understand the rostrocaudal extent of this phenotype (**Supplemental Fig. S6**).



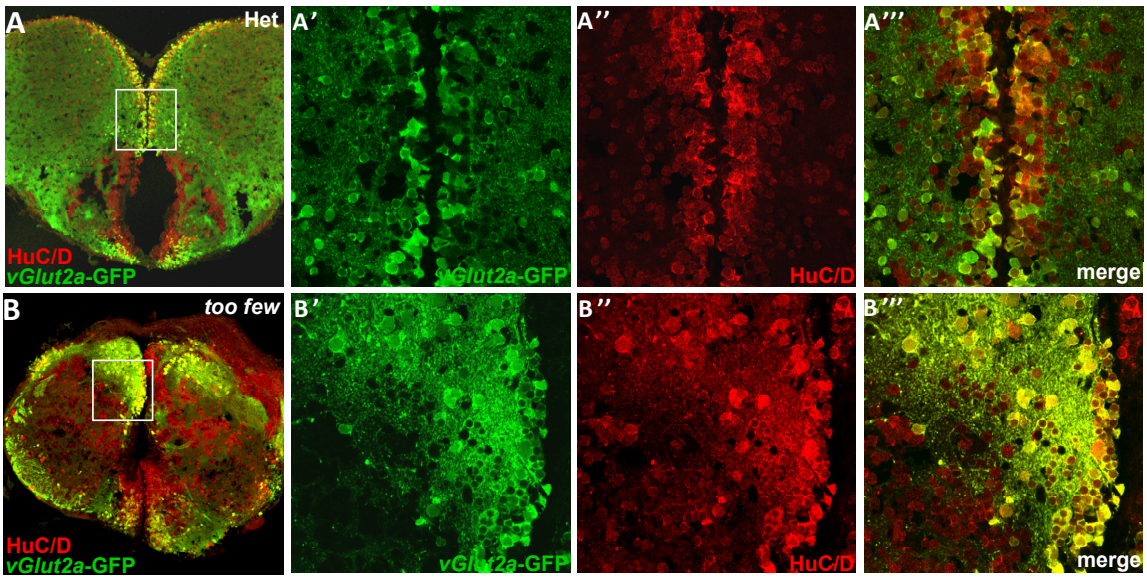
Supplemental Figure S6. Serial sections through adult *too few* homozygous mutant telencephalon, with triple-labeling for *fezf2*-GFP/BLBP/PCNA.
Serial sections through rostrocaudal extent of the adult *too few* mutant telencephalon, showing distribution of *fezf2*-GFP+ cells as well as PCNA (marker of proliferation) and BLBP (marker of neural stem cells) (20X confocal projection images). Abbreviations: D dorsal telencephalon, V ventral telencephalon, OB olfactory bulb.

Further analysis of the adult *too few* mutant telencephalon revealed an overall increase in the number of HuC/D+ neurons adjacent to *fezf2*-GFP+ radial glial-like cells of the telencephalic VZ (n=3 heterozygote and 3 homozygote brains; $p<0.01$; **Fig. 3 E-G**). Serial sections through adult mutant telencephalon are provided to better understand the rostrocaudal extent of this phenotype (see **Supplemental Fig. S7**). We noticed that the HuC/D+ neurons appear to be smaller in size in the mutant, indicating that these neurons may not be fully normal.



Supplemental Figure S7. Serial sections through adult *too few* homozygous mutant telencephalon, with double-labeling for *fezf2*-GFP and HuC/D.
Serial sections through rostrocaudal extent of the adult *too few* mutant telencephalon, showing distribution of *fezf2*-GFP+ cells as well as HuC/D+ neurons (20X confocal projection images).
Abbreviations: D dorsal telencephalon, V ventral telencephalon, OB olfactory bulb.

To determine whether the observed increase in neurons near the VZ is due to an increase in adult telencephalic neurogenesis, we administered an extended pulse of BrdU (3 injections per day for 2 days), followed by a 2-week chase period. As shown in **Fig. 3H-J**, we found a significant increase in the number of adult-born neurons adjacent to *fezf2*-GFP+ cells of the telencephalic VZ in the homozygous mutant, compared with heterozygote siblings (n=3 heterozygote and 3 homozygote brains; $p<0.05$). We also found that the neuronal build-up in the adult *too few* homozygous mutant telencephalon is likely a build-up of glutamatergic neurons, as indicated by analysis of the *vglut2a*-GFP transgenic line crossed to the mutant background (n=3 heterozygote and 3 homozygote brains; **Supplemental Fig. S8**).



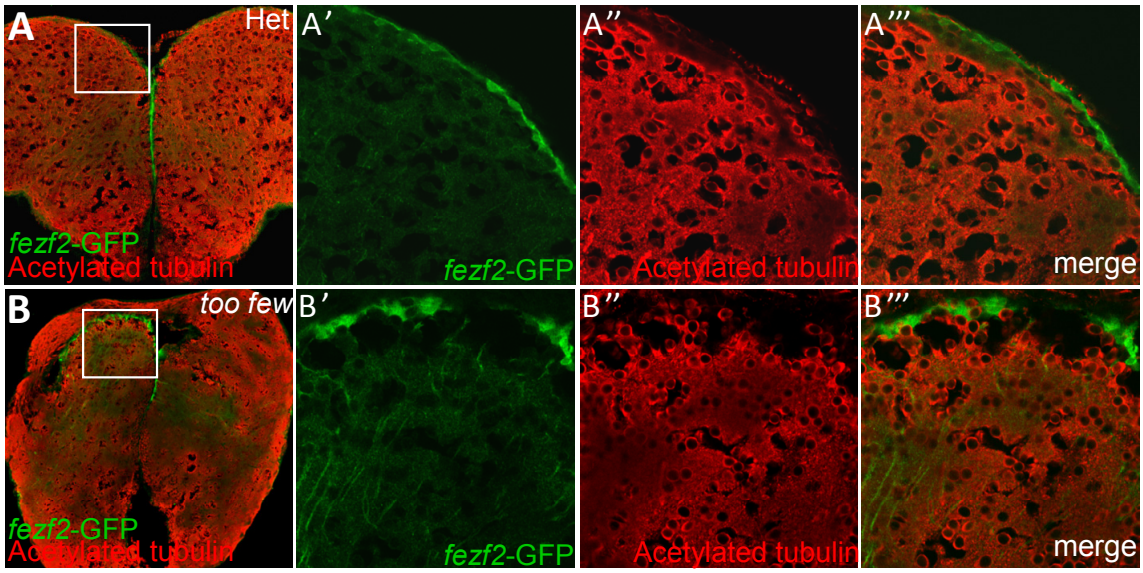
Supplemental Figure S8. Build-up of neurons in adult *too few* homozygous mutant telencephalon is likely a build-up of *vglut2a*-GFP+ glutamatergic neurons, as indicated by analysis of *too few* mutants crossed to the *vglut2a*-GFP transgenic line.
(A) Coronal section through *too few* heterozygous mutant telencephalon, with double-labeling for *vglut2a*-GFP and HuC/D (neuronal marker) (20X magnification confocal projection image) (n=3 heterozygote brains). (A'-A''') 100X magnification of boxed region in (A) showing *vglut2a*-GFP+/Hu+ cells. (B) Coronal section through *too few* homozygous

mutant telencephalon, with double-labeling for *vglut2a*-GFP and HuC/D (20X magnification confocal projection image) (n=3 homozygote brains). (B'-B''') 100X magnification (single confocal z-section) of boxed region in (B) shows build-up of *vglut2a*-GFP+/Hu+ cells.

Taken together, these findings suggest that *fezf2* may be important to maintain telencephalic radial glial-like cells in a non-proliferative state, possibly limiting their neurogenic potential.

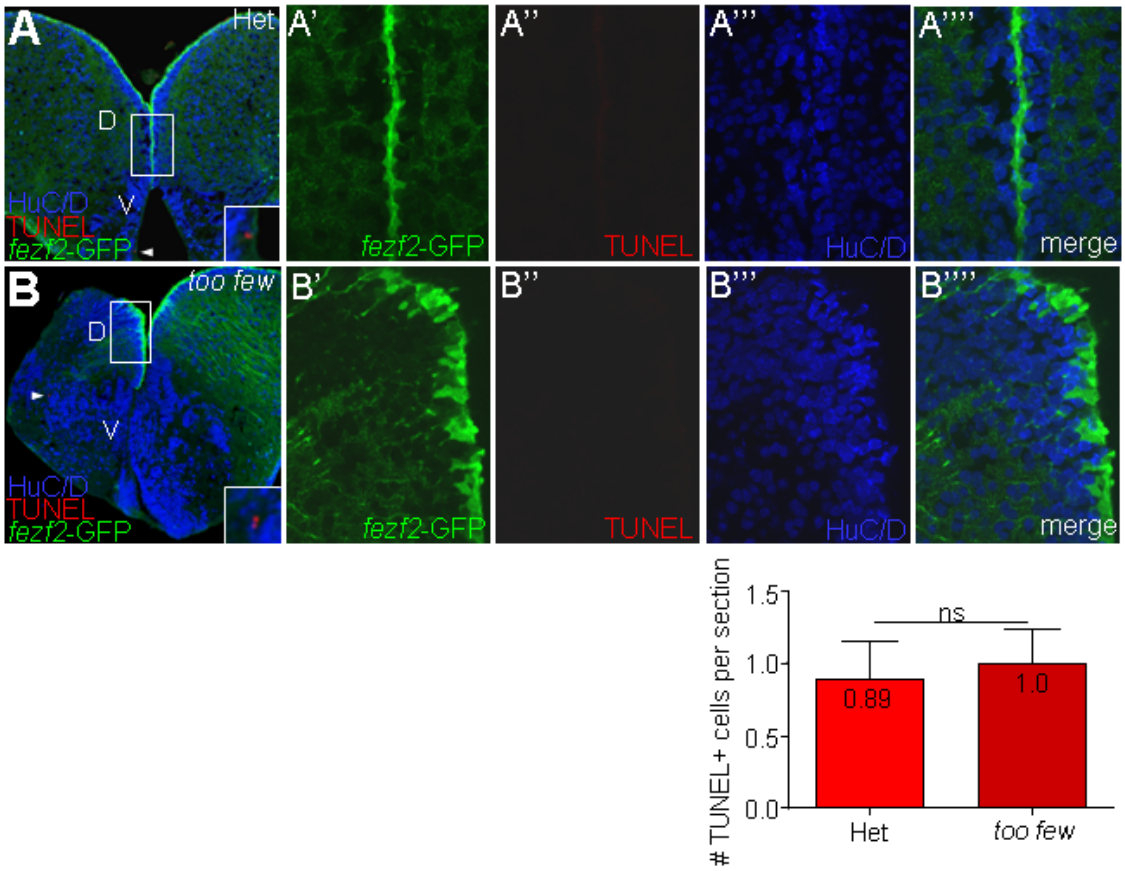
Decrease in neuropil may account for smaller telencephalon in adult too few mutant

Despite increases in proliferation and adult neurogenesis in the mutant telencephalon, we find that the mutant telencephalon is smaller in size. One possibility is that this decrease in size is due to a decrease in neuropil within the telencephalon. To test this idea, we performed staining for acetylated-tubulin (stains neuronal processes) and observed a decrease in acetylated-tubulin labeling in the mutant telencephalon (n=3 heterozygote and 3 homozygote brains; **Supplemental Fig. S9**), suggesting that neurons may not be maturing properly. This is consistent with the observation that the neurons in the mutant telencephalon appear to be smaller in size as well. We noticed that *fezf2*-GFP+ radial processes seem to be taking over some of the territory lost by the neuropil in the homozygous mutant telencephalon (**Supplemental Fig. S9**).



Supplemental Figure S9. Decrease in neuropil may account for smaller telencephalon in adult *too few* mutant.
(A) Coronal section through *too few* heterozygous mutant telencephalon, with double-labeling for *fezf2*-GFP and acetylated tubulin to label neuronal processes (n=3 heterozygote brains). (A'-A''') 100X magnification (single confocal z-section) of boxed region in (A). (B) Coronal section through *too few* homozygous mutant telencephalon, with double-labeling for *fezf2*-GFP and acetylated tubulin (n=3 homozygote brains). (B'-B''') 100X magnification (single confocal z-section) of boxed region in (B). There appears to be a decrease in acetylated tubulin labeling in the homozygous mutant telencephalon compared with heterozygote sibling controls, and *fezf2*-GFP+ radial processes seem to be taking over territory lost by the neuropil.

A smaller telencephalon in the *too few* homozygous mutant may also result from an increase in cell death. We performed TUNEL staining to test this possibility. As shown in **Supplemental Fig. S10**, we found no difference in the number of TUNEL+ cells in the mutant adult telencephalon, compared with heterozygote sibling controls (n=3 heterozygote and 3 homozygote brains). This indicates that at least in the adult, increased cell death does not account for the smaller telencephalon.

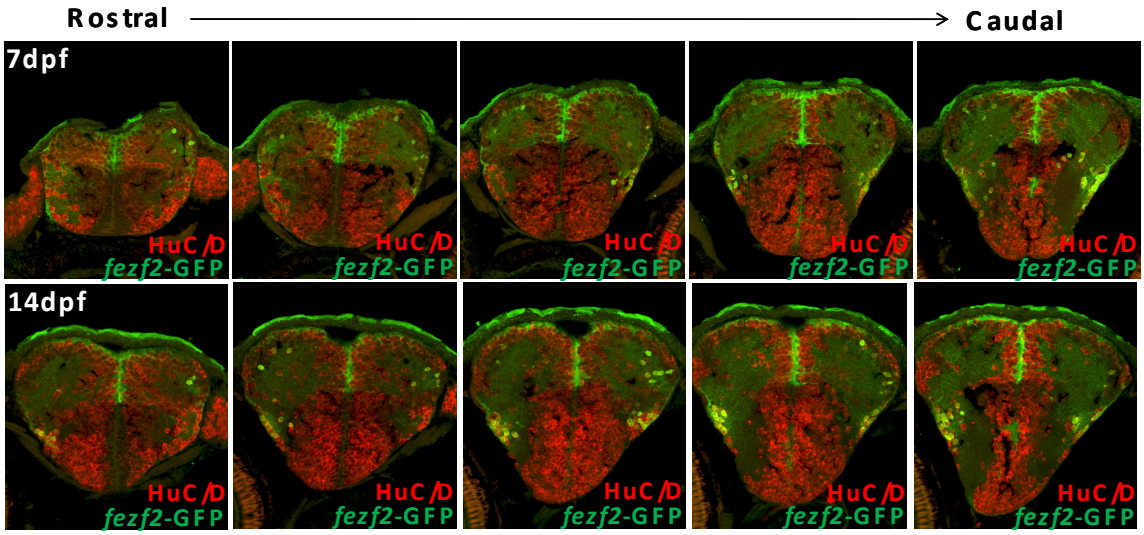


Supplemental Figure S10. Differences in cell death do not account for smaller telencephalon in adult *too few* mutant.

(A) Coronal section through *too few* heterozygous mutant telencephalon, with triple-labeling for *fezf2*-GFP, TUNEL, and HuC/D. Only about one TUNEL+ cell was detected in any given section, indicated by arrowhead and enlarged in the inset (n=3 heterozygote brains). (A'-A'''') 100X magnification (single confocal z-section) of boxed region in (A) shows no TUNEL+ cells near the midline ventricular zone of the dorsal telencephalon. (B) Coronal section through *too few* homozygous mutant telencephalon (sibling) shows no differences in TUNEL+ staining, indicated that there are no differences in cell death in the *too few* homozygous mutant. Rare TUNEL+ cells are indicated by arrowhead and enlarged in the inset (n=3 homozygote brains). (B'-B'''') 100X magnification (single confocal z-section) of boxed region in (B) shows no TUNEL+ cells near the midline ventricular zone of the dorsal telencephalon. Quantification of the number of TUNEL+ cells per section indicates that there is no significant difference between heterozygote and homozygote brains. Abbreviations: D dorsal telencephalon, V ventral telencephalon.

No major differences observed in too few mutant telencephalon at the larval stage, suggesting that the phenotype occurs largely at the late-larval to adult stage

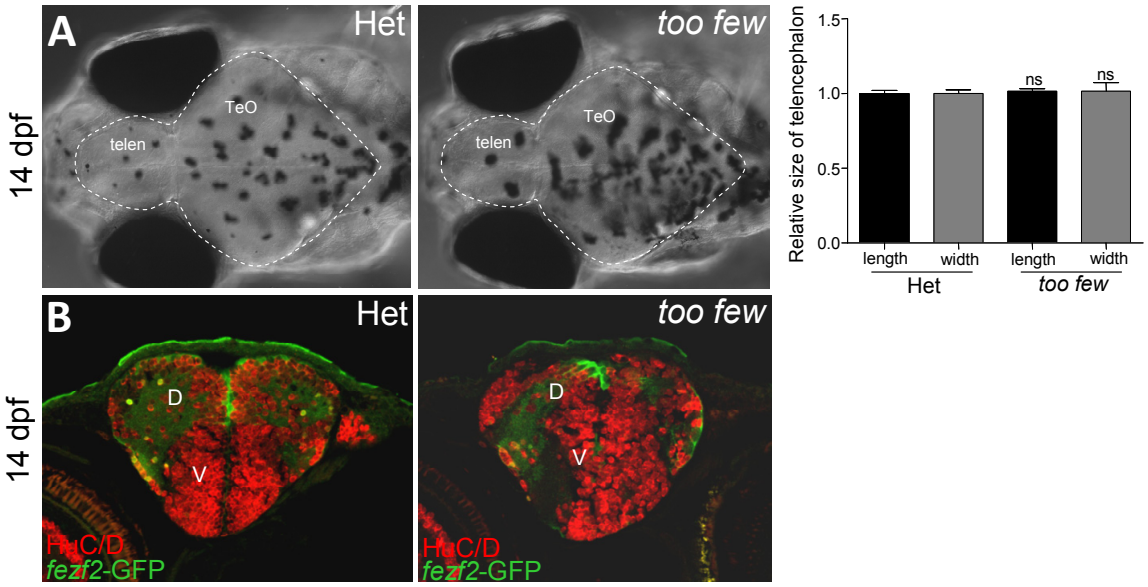
Since the *too few* mutant has a mutation in the *fezf2* gene all throughout development, it is possible that adult phenotypes are due, at least in part, to a role for *fezf2* during development. Along these lines, we have found that *fezf2*-GFP also labels radial glial cells at the larval stage (7dpf and 14dpf; **Supplemental Fig. S11**).



Supplemental Figure S11. *fezf2*-GFP labels radial glial cells at the larval stage.
Serial sections through 7dpf and 14dpf larval zebrafish showing *fezf2*-GFP expression throughout the rostrocaudal extent of the telencephalon, with double-labeling for HuC/D (neuronal marker). *Fezf2*-GFP expression labels radial glial cells at these larval stages (40X magnification, confocal projection images).
These samples of larval *fezf2*-GFP expression are in the *too few* heterozygous mutant background.

To determine whether *fezf2* functions during telencephalic development, we analyzed homozygous mutants (and heterozygote sibling controls) at the larval stage (14dpf). Though no differences were observed in the overall size of the telencephalon at this stage (n=3 heterozygote and 4 homozygote larvae; **Supplemental Fig. S12**), analysis on

sections reveals changes in the overall organization of the telencephalon, including a shift in the *fezf2*-GFP+ progenitor domain and an expansion of the subpallium, which are observed at the adult stage as well.



Supplemental Figure S12. No differences are observed in the overall size of the telencephalon at larval stage (14dpf), but analysis on sections reveals changes in organization of the telencephalon.
(A) Dorsal view of 14dpf (days post fertilization) larval *too few* homozygote and heterozygote siblings shows no significant difference in the overall size of the telencephalon (relative to the rest of the brain) at this stage (n=3 heterozygote larvae; n=4 homozygote larvae). (B) Analysis on sections, with labeling for *fezf2*-GFP and HuC/D reveals, reveals changes in organization of the telencephalon. *Fezf2*-GFP+ progenitor domain seems to be shifted more dorsally, and the ventral telencephalon (subpallium) is expanded, as we have noticed at the adult stage as well.
Abbreviations: telen telencephalon, TeO optic tectum, D dorsal telencephalon, V ventral telencephalon.

These observations leave the possibility that *fezf2* may be playing a role in developmental patterning (or morphogenesis) of the telencephalon, such that the observed increases in proliferation and neurogenesis in the adult mutant telencephalon may be indirect

(secondary) effects stemming from changes during development to the overall environment of the telencephalon.

Transplantation of fezf2-GFP+ mutant cells into wildtype hosts reveals a cell-autonomous role for fezf2 in regulating the proliferation of telencephalic radial glial cells

To determine whether changes in the environment of the mutant telencephalon are responsible for the observed increases in proliferation, and to determine whether *fezf2* functions autonomously to regulate the proliferation of telencephalic radial glial cells, *fezf2*-GFP+ heterozygous and homozygous mutant cells were transplanted (from *fezf2*-GFP+ donors) into wildtype hosts at the embryonic stage, and these hosts were grown to adulthood (3 months old) for analysis. Transplantations were performed by obtaining 40 cells from 3-4hpf (hours post fertilization) stage *fezf2*-GFP/*too few* donors and placing them into the animal pole of equal age wildtype hosts. Analysis of hosts transplanted with *fezf2*-GFP+ heterozygous mutant cells indicates that transplanted cells persist to the adult stage. Moreover, transplanted cells are found as clusters of *fezf2*-GFP+/Sox3+ radial glial-like cells, and are more proliferative than neighboring control (*fezf2*-expressing wildtype) cells, as indicated by PCNA labeling (marker of proliferation) (n=3 brains; **Fig. 4 A-C**, and **Supplemental Figure S13**). No major differences were observed in the number of HuC/D+ neurons near *fezf2*-GFP+ heterozygous transplanted cells (**Fig. 4 A-B**, and **Supplemental Fig. S13**).

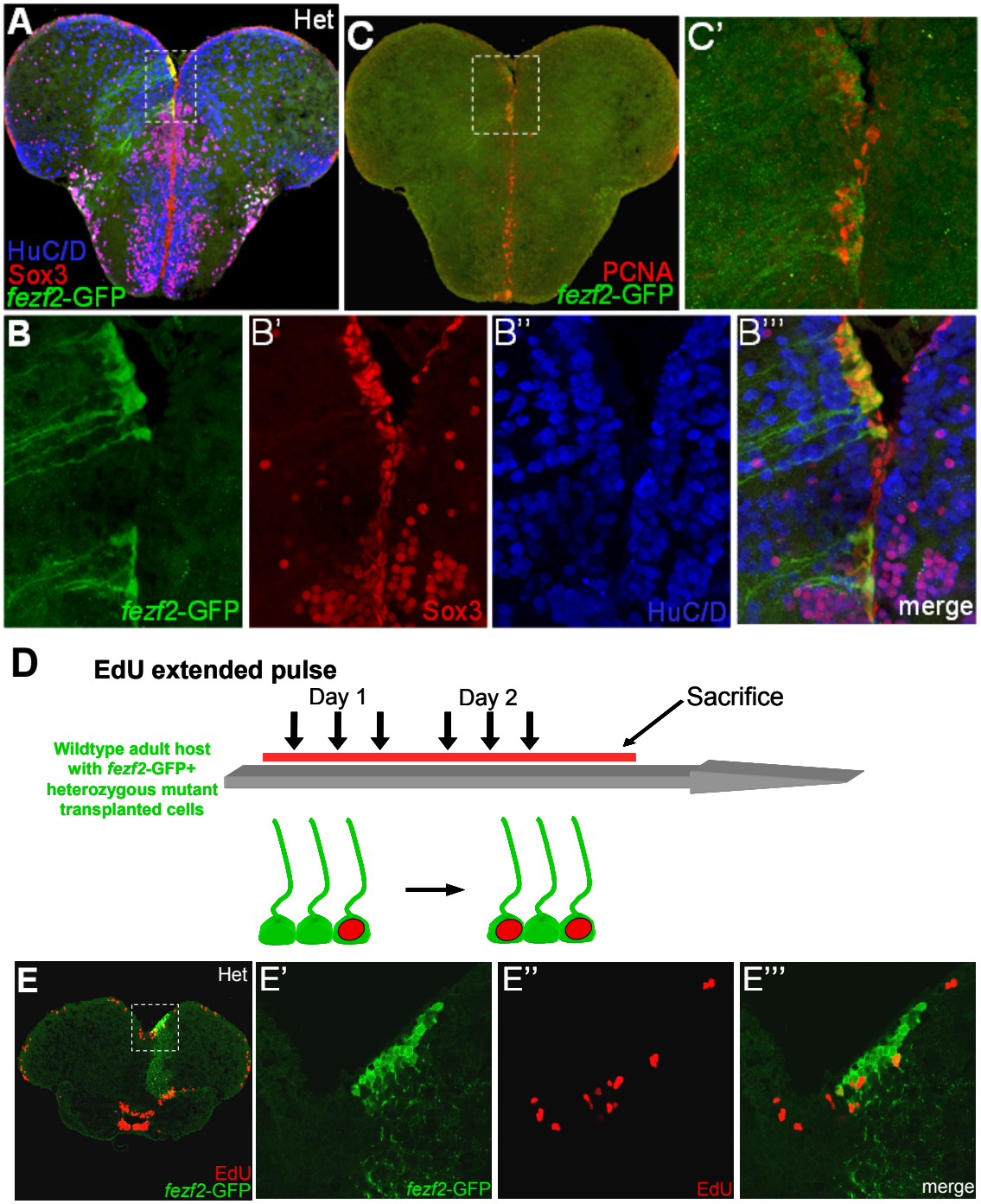
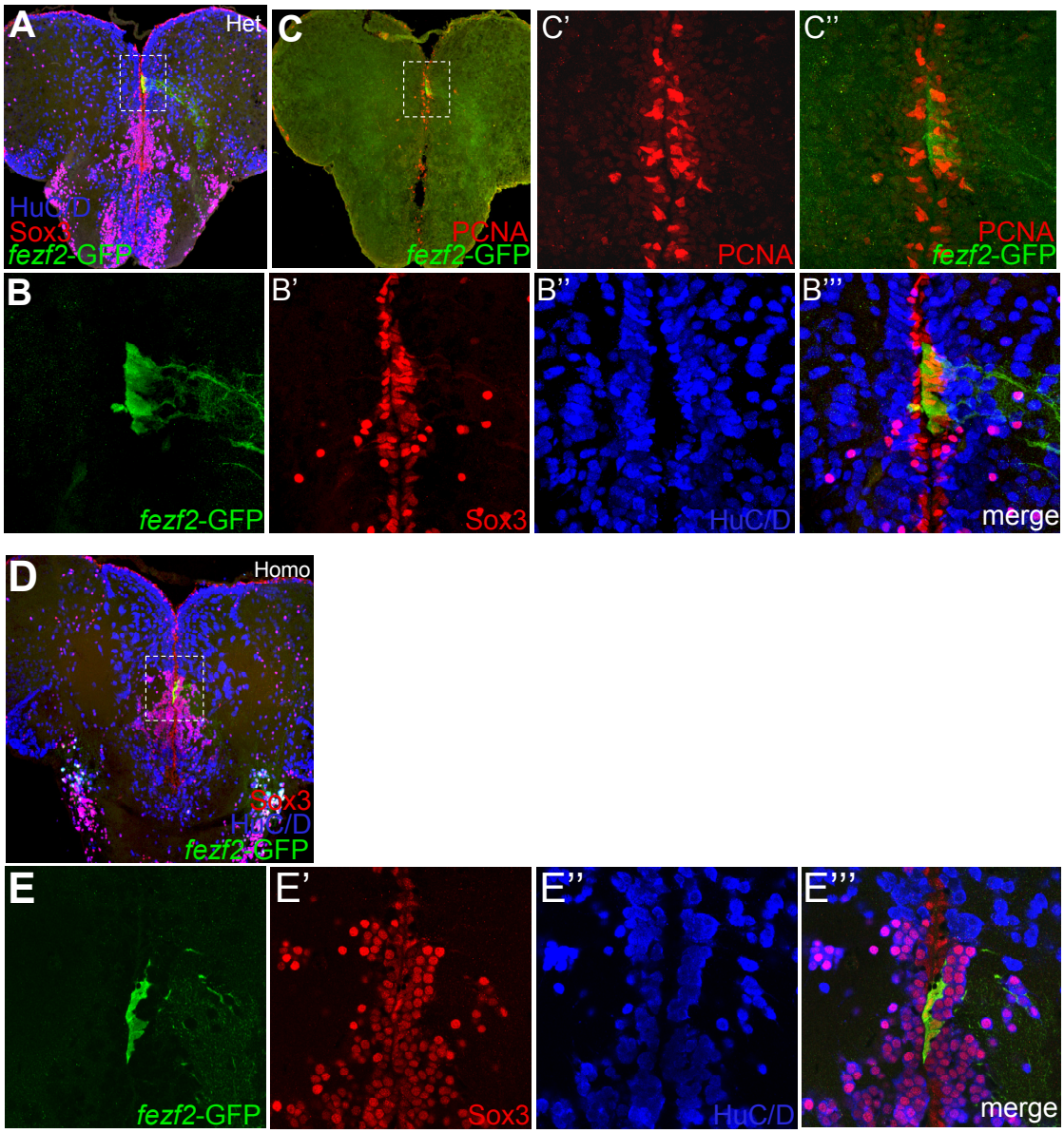


Figure 4. Transplantation of *fezf2*-GFP+/*too few* mutant cells into wildtype hosts at the embryonic stage suggests that *fezf2* may function cell-autonomously in regulating the proliferation of radial glial cells of the telencephalic ventricular zone, as visualized at the adult stage. (A) Coronal section through adult wildtype telencephalon showing the presence of *fezf2*-GFP+/*too few* heterozygous mutant transplanted cells within the dorsal telencephalon, with triple-labeling for *fezf2*-GFP/Sox3/Hu (20X magnification, confocal

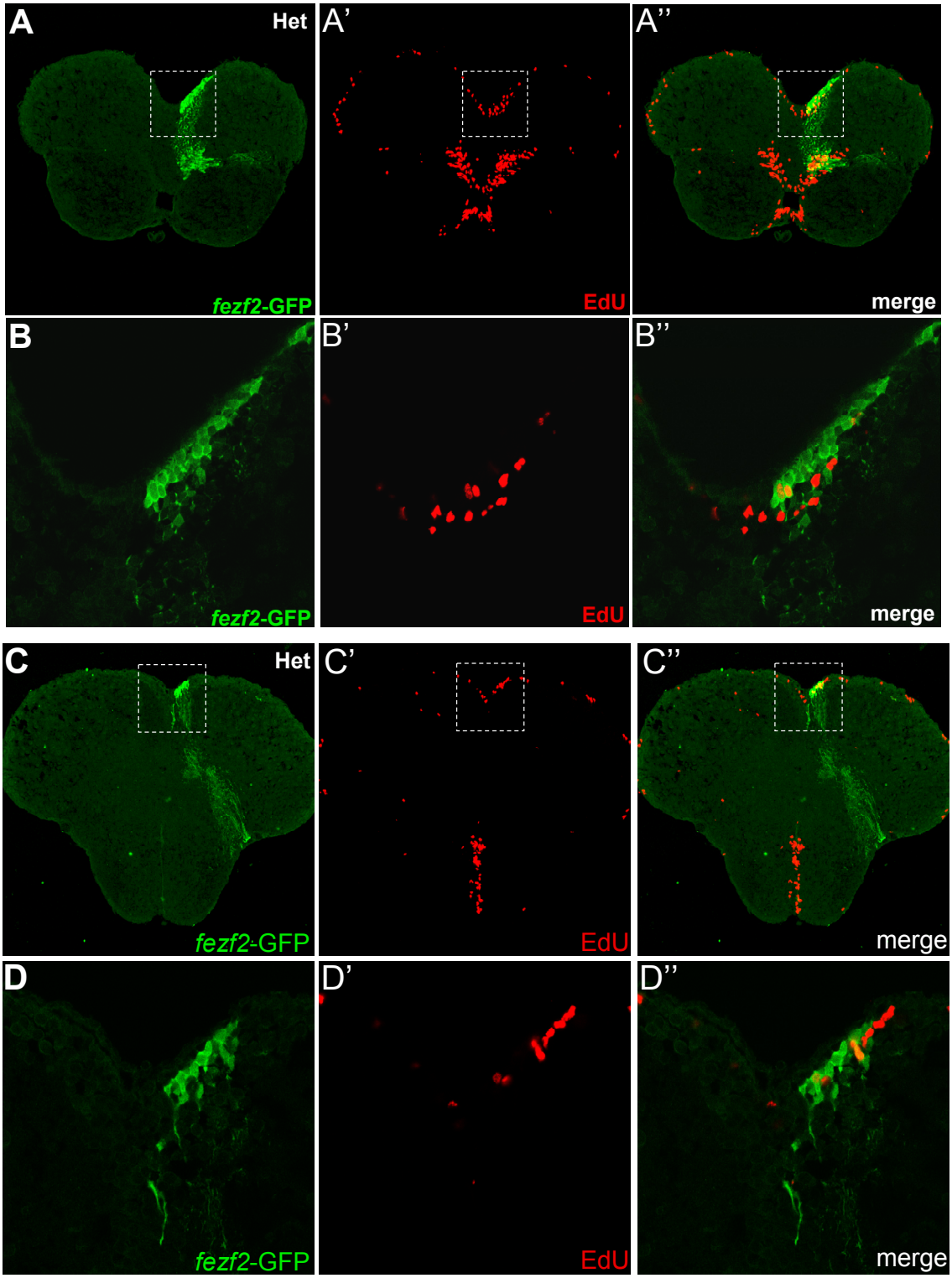
projection image). (B-B''') 100X magnification (confocal projection image) of boxed region in (A) shows a build-up of *fezf2*-GFP+/Sox3+ mutant transplanted cells, compared to untransplanted control hemisphere (containing wildtype Sox3+ radial glial-like cells). (C) Analysis of adjacent sections from the same brain indicates that *fezf2*-GFP+ mutant transplanted cells are proliferating more rapidly than neighboring untransplanted (wildtype) cells (20X magnification, confocal projection image). (C') 100X magnification (single confocal z-section) of boxed region in (C) shows increase in PCNA labeling in *fezf2*-GFP+ transplanted clusters compared to neighboring untransplanted (wildtype) cells (n=3 host brains with heterozygote transplanted cells). (D) Schematic diagram showing EdU extended pulse (2 days, 3 injections per day) in wildtype adult hosts containing *fezf2*-GFP+ heterozygous mutant transplanted cells to label cells in S-phase. (E) Coronal section through telencephalon, with double-labeling for *fezf2*-GFP and EdU (20X magnification, confocal projection image). (E'-E''') 100X magnification (single confocal z-section) of boxed region in E showing colocalization of *fezf2*-GFP+ cells with EdU. *Fezf2*-GFP+ heterozygous mutant transplanted cells appear to be more actively proliferating than neighboring regions with untransplanted (wildtype) cells.

As PCNA labeling requires acid treatment for antigen retrieval, a substantial amount of the *fezf2*-GFP signal is destroyed, making it difficult to determine whether the *fezf2*-GFP+ cells themselves are actually proliferating with cellular resolution. To address this, an extended pulse of EdU (3 injections per day for 2 days) was administered (schematized in **Fig. 4 D**), and results show that *fezf2*-GFP+ heterozygous mutant transplanted cells colocalize with EdU and seem to be proliferating more than cells in neighboring (untransplanted) regions (**Fig. 4 E**, and **Supplemental Fig. S14**), further supporting a cell-autonomous role for *fezf2* in suppressing the proliferation of telencephalic radial glial cells. Additional EdU-injected samples with heterozygote transplanted cells are being analyzed. Furthermore, to control for the possibility that the transplantation procedure itself may have resulted in the observed heterozygous phenotype, transplantation of *fezf2*-GFP+ wildtype cells into wildtype hosts are in progress.



Supplemental Figure S13. Examples from additional host brains, with *fezf2*-GFP+/*too few* heterozygous and homozygous mutant transplanted cells.

(A) Host with *fezf2*-GFP+ heterozygous mutant transplanted cells, with triple-labeling for *fezf2*-GFP, Sox3, and HuC/D (20X magnification confocal projection image). (B-B''') 100X magnification (single confocal z-section) of boxed region in (A) shows cluster of *fezf2*-GFP+/Sox3+ cells. (C-C'') Double-labeling of an adjacent section with *fezf2*-GFP and PCNA reveals an increase in proliferation in the region of the transplanted cluster (n=3 brains). (D) Host with *fezf2*-GFP+ homozygous mutant transplanted cells, with triple-labeling for *fezf2*-GFP, Sox3, and HuC/D (20X magnification confocal projection image). Only one small group of cells, shown here, was found out of all brains analyzed with homozygous mutant transplanted cells (n=3 brains). (E-E''') 100X magnification (single confocal z-section) of boxed region in (D).



Supplemental Figure S14. Additional examples of EdU extended pulse labeling from host with *fezf2*-GFP+ heterozygous mutant transplanted cells reveals an increase in proliferation at the region of the transplanted cells.

(A-A'') Example of transplanted cells within the more rostral part of the dorsal telencephalon with labeling for *fezf2*-GFP and EdU (20X magnification confocal projection image). (B-B'') 100X magnification (single confocal z-section) of boxed region in (A-A'') shows some *fezf2*-GFP+/EdU+ cells, as well and EdU+ cells that are *fezf2*-GFP negative in close proximity, which may represent intermediate progenitor cells derived from proliferating *fezf2*-GFP+ cells, although we cannot determine this without a lineage tracer.
(C-C'') Example of transplanted cells within the more caudal part of the dorsal telencephalon with labeling for *fezf2*-GFP and EdU (20X magnification confocal projection image). Again notice an increase in EdU labeling at the site of heterozygous mutant transplanted cells. (D-D'') 100X magnification (single confocal z-section) of boxed region in (C-C'') shows *fezf2*-GFP+/EdU+ cells, as well as EdU+/*fezf2*-GFP negative cells in close proximity.

Since we did not observe any obvious phenotype in the adult *too few* heterozygous mutant telencephalon, we were surprised to see a phenotype with *too few* heterozygous mutant transplanted cells. In our analysis of *fezf2*-GFP+ *too few* homozygous mutant transplanted cells (n=3 brains), we only found one small *fezf2*-GFP+ cluster in one brain (see **Supplemental Fig. S13**), and no *fezf2*-GFP+ cells in any of the other brains. These findings suggest that *fezf2*-GFP+ homozygous mutant transplanted cells may be dying or are depleted during development.

Discussion

In this study, we investigated a functional role for Fezf2 in the proliferation and differentiation of neural progenitor cells in the adult zebrafish telencephalic VZ, building on our previous finding that *fezf2* is expressed in radial glial-like cells that label with markers of neural stem cells and proliferation within the adult zebrafish telencephalon (Berberoglu et al., 2009).

Addressing neural stem cell properties of fezf2-GFP+ cells

Until now, self-renewal of adult neural stem cells has been assessed largely *in vitro* using the neurosphere assay, in which the formation of "neurospheres," or clusters of neural progenitor cells, form from a single neural stem cell. The formation of secondary neurospheres from dissociated primary neurospheres provides evidence for self-renewal. Thus far, few studies have assessed self-renewal *in vivo*. In this study, we provide *in vivo* evidence that *fezf2*-GFP+ cells are capable of self-renewal.

Though *fezf2*-GFP+ cells have the ability to self-renew, it is important to remember that a majority of these cells are non-proliferating at any given time. It is thus possible that at least some *fezf2*-GFP+ cells may represent ependymal-like cells in the adult zebrafish telencephalon. From our experiments, we can only conclude that some *fezf2*-GFP+ cells may have the ability to self-renew; however, this may not be a characteristic property of all *fezf2*-GFP+ cells. It is also important to realize that we do not know if the original cell labeled was *fezf2*-expressing in these experiment, as we are only observing results at the end-point.

Additionally, we used BrdU as a lineage tracer to show that *fezf2*-GFP+ cells may be able to give rise to neurons in the adult zebrafish telencephalon, and that these neurons are likely to be glutamatergic. Though BrdU has been used as a lineage tracer for many years before the development of current technologies, BrdU lineage tracing in our experiments does not provide definitive evidence that *fezf2*-GFP+ cells can give rise to glutamatergic neurons, as some BrdU+/*fezf2*-GFP- cells are present with an extended pulse of BrdU (3 injections per day for 2 days), indicating that the initial population of progenitor cells labeled with BrdU is not made up exclusively of *fezf2*-GFP+ cells. Additionally, some BrdU+/*vglut2a*-GFP-negative cells are present after the 2-week chase period, suggesting that progenitor cells labeled with the initial pulse of BrdU are capable of giving rise to *vglut2a*-GFP+ cells, but can also give rise to other cell types. More advanced methods, such as CreER lineage tracing of *fezf2*-expressing cells, can be employed in the future to directly establish this connection.

During the course of our study, an article was published in which viral vectors were successfully used to transduce these radial glial-like cells of the adult zebrafish dorsal telencephalic ventricular zone (the same cells that we are studying as well), confirming that these cells can self-renew and give rise to neurons, and providing further evidence that these cells are likely to be adult neural stem cells (Rothenaigner et al., 2011).

Fezf2 regulates the proliferation and differentiation of adult neural progenitor cells

Our analysis of the adult *too few* (Fezf2) mutant telencephalon leads us to a model whereby Fezf2 maintains adult zebrafish telencephalic radial glial-like cells in a non-proliferative state, which sheds light for the first time on a role for Fezf2 in regulating

adult neural progenitor cells. Such a role for Fezf2 is critical to maintain proper cell numbers and prevent over-proliferation.

One of the caveats of the mutant analysis is that our adult phenotype could be confounded by a role for *fezf2* in the telencephalon during development. To directly test whether Fezf2 plays a role in regulating the proliferation and differentiation of adult neural progenitor cells, it is ideal to employ conditional and inducible strategies to block Fezf2 function. Unfortunately, conditional approaches (i.e. floxed alleles, CreER, viral delivery) that are routinely used in other model systems such as mouse are quite difficult in zebrafish using current technologies.

Nevertheless, we were able to show that changes in the structure, or environment, of the telencephalon during development are not likely to account for the observed increase in proliferation of *fezf2*-GFP+ cells at the adult stage, as transplantation of *fezf2*-GFP+ mutant cells into wildtype hosts suggests a cell-autonomous role for *fezf2* in regulating the proliferation of *fezf2*-GFP+ cells (see below). Control experiments in which *fezf2*-GFP+ wildtype cells are transplanted into wildtype hosts will be necessary to conclusively establish this.

Fezf2 functions cell-autonomously to suppress the proliferation of telencephalic radial glial cells

Transplantation of *fezf2*-GFP+ heterozygous cells into wildtype hosts confirms that the increase in proliferation that we observed in the adult *too few* mutant telencephalon is due to a lack of Fezf2 function within the radial glial cells, rather than simply a secondary effect of a changed brain environment. This cell-autonomous role of Fezf2 in

suppressing the proliferation of telencephalic radial glial cells parallels our findings in the adult *too few* mutant telencephalon, and further establishes a role for Fezf2 in suppressing the proliferation of adult neural progenitor cells. However, since cells were transplanted at the embryonic stage, there is still a possibility that Fezf2 function in embryonic/larval radial glial cells may have influenced the adult radial glial-like cells. This is a possibility that we cannot exclude, and future experiments using conditional approaches at the adult stage can be employed to address this.

Additionally, we were surprised to see a phenotype with heterozygous transplanted cells, as the adult *too few* heterozygote telencephalon appears normal. It is possible that with only a small number of heterozygote transplanted cells among many surrounding wildtype cells, we are able to see subtle phenotypic changes that we did not observe in the adult *too few* heterozygote telencephalon. Alternatively, since the phenotype of our heterozygous transplanted cells is quite robust, it is possible that heterozygous mutant cells surrounded by wildtype neighbors act differently based on cell-cell communication and mechanisms which we do not fully understand, resulting in amplification of the phenotype in these cells. The fact that most homozygous transplanted cells did not persist to adulthood suggests that these cells may be dying or are outcompeted during development.

Nevertheless, our results obtained with *fezf2*-GFP+ heterozygous transplanted cells parallel our finding in the adult *too few* mutant telencephalon, and further support a role for *fezf2* in suppressing the proliferation of telencephalic radial glial cells.

Understanding the functional role of adult neurogenesis within the Dm region

Our findings point to an increase in neurogenesis within the adult *too few* mutant telencephalon, adjacent to *fezf2*-GFP+ cells. This region of *fezf2* expression and active adult neurogenesis is the medial part of the dorsal telencephalon (Dm), which is thought to be similar to the mammalian amygdala. Enhanced adult neurogenesis within the amygdala region could have important functional implications in terms of understanding what these new neurons may actually be doing. An increase in the production of neurons, presumably glutamatergic, within the adult *too few* mutant amygdala could perhaps result in augmentation of amygdalar function, which may be manifested by increases in fear or anxiety, for example.

Thus, studying behavior of the *too few* mutant could provide important insight in understanding the function of new adult neurons. However, it is important to keep in mind that the *too few* mutant also has defects in hypothalamic dopamine neurons at the adult stage (Rink and Guo, 2004), making it difficult to attribute any observed behavioral phenotypes to only the telencephalon.

Mechanism of fezf2 action in adult neural progenitor cells

If Fezf2 plays a role in maintaining adult zebrafish telencephalic radial glial-like cells in a non-proliferative state, there is interest in understanding the mechanism by which this occurs. One possibility is that Fezf2 interacts with known signaling pathways that have been previously implicated in the proliferation and differentiation of neural stem cells, such as Notch, Shh, Wnts, Fgfs, or BMPs, among others. Two recent studies have shown that Notch signaling is at least one likely mechanism by which Fezf2 functions in neural

progenitor cells (Shimizu et al., 2010; Weng et al., 2010). Furthermore, it was recently demonstrated that Notch signaling controls the balance between quiescence and proliferation of radial glial-like cells in the adult zebrafish telencephalon (Chapouton et al., 2010). As such, it is possible that Notch signaling may be a mechanism of Fezf2 action in our system as well. Additional candidates such as Shh signaling, Wnts, Fgfs, and BMPs can also be explored in future studies.

Is expression of fezf2 in adult neural stem cells evolutionarily conserved?

Given that *fezf2* is expressed in radial glial-like cells of the adult zebrafish telencephalic VZ, which display properties of adult neural stem cells, we asked whether *fezf2* is expressed in adult neural stem cells in mouse. A look into a few online databases provides evidence that *fezf2* is likely to be expressed in adult neural stem cells in mouse, making *fezf2* expression conserved among vertebrates. Perhaps most striking are images of *fezf2*-GFP BAC transgenic mice (GENSAT database), which confirm expression in cells with radial glial morphology within the adult dentate gyrus (most likely radial astrocytes), and cells with the morphology of B cells in the SVZ. *In situ* hybridization for *fezf2* mRNA reveals low levels of expression in the dentate gyrus of the hippocampus as well as in the SVZ of adult mice (Allen Brain Atlas). This conserved expression of *fezf2* opens the door to future studies aimed at understanding the role of Fezf2 in the regulation of adult neural stem cells and adult neurogenesis in mammalian systems.

Materials & Methods

Zebrafish strains and maintenance

3- to 14-month old adult zebrafish (*Danio rerio*) were used in this study, including wildtype zebrafish of the AB strain, *fezf2*-GFP transgenic fish (Berberoglu et al., 2009), *vglut2a*-GFP transgenic fish (Bae et al., 2009), and *too few* mutant zebrafish. Adult and larval *too few* mutant zebrafish were identified by genotyping for the missense mutation (Levkowitz et al., 2003). All procedures using animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco.

BrdU and EdU labeling

Fish were anesthetized with Tricaine and injected intraperitoneally (I.P.) with a 10mM solution of Bromodeoxyuridine (BrdU) diluted in PBS twice with a 4-hour interval (pulse) or three times per day (with 4-hour interval between injections) for a period of 2 days (extended pulse), and were then sacrificed within 24 hours (for acute labeling) or allowed to survive for longer periods of time for pulse-chase experiments, as indicated in the text. The same parameters were used for injection of a 10mM solution of EdU. Fish were anesthetized, perfused with PBS and 4% paraformaldehyde (Pfa), and were fixed overnight. Brains were dissected out the following day, followed by cryoprotection overnight (30% sucrose in PBS). Brains were embedded in OCT, and cryosectioning was performed (18 micron-thick). EdU click chemistry reaction was performed according to Invitrogen instructions (Click-iT EdU Alexa Fluor 594 Imaging Kit).

Immunohistochemistry for BrdU was performed as described below (note modification to protocol for BrdU labeling or for BrdU and EdU double-labeling).

Immunohistochemistry

Adult zebrafish were processed as described above, followed by cryoprotection overnight (30% sucrose in PBS). Brains were embedded in OCT, and cryosectioning was performed (18 micron-thick). Cryosections were washed in PBS (3X, 10 minutes), followed by washes in PBS + 0.5% Triton (2X, 5 minutes), and PBS (3X, 10 minutes). Cryosections were blocked in 3% BSA in PBS for 30 minutes and were incubated with primary antibodies (diluted in 3% BSA) overnight at 4 degrees C. The following day, sections were washed in PBS (3X, 10 minutes), followed by washes in PBS + 0.1% Triton (2X, 5 minutes). Sections were then incubated with secondary antibodies (diluted in PBS + 0.1% Triton) for 2 hours, followed by washes with PBS (6X, 10 minutes). Slides were then mounted (using Dako fluorescent mounting medium) and coverslipped. Modification to protocol: For BrdU and PCNA labeling, the sections were incubated in 4N HCl for 20 minutes at room temperature and washed with PBS (3X, 10 minutes) before washes with PBS + 0.5% Triton. For BrdU labeling without EdU, rat anti-BrdU antibody was used (1:2000, Abcam). For BrdU and EdU double-labeling, mouse anti-BrdU (1:600, Sigma) was used, as this antibody does not cross-react with EdU (see below).

The following primary antibodies were used: anti-GFP (chicken, 1:2500, Abcam), anti-HuC/D (mouse, 1:1000, Invitrogen), anti-BLBP (rabbit, 1:400, Abcam), anti-Sox3 (rabbit, 1:1000, a gift from Dr. M. Klymkowsky), anti-PCNA (mouse, 1:500, Dako), anti-

acetylated tubulin (mouse, 1:2000, Sigma), anti-BrdU (rat, 1:2000, Abcam), anti-BrdU (mouse, 1:600, Sigma). Alexa secondary antibodies were used (1:200 dilution), with 488/568 used for double-labeling and 488/568/647 used for triple-labeling.

TUNEL staining for cell death

TUNEL labeling for cell death was performed as described in Roche *In Situ* Cell Death Detection Kit, TMR red. Slides were first washed 3X, 10 minutes in PBS, followed by washes in PBS + 0.5% Triton (2X, 5 min.), and PBS (3X, 10 min.). Slides were then incubated with TUNEL reaction mixture. For double and triple-labeling, immunohistochemistry was subsequently performed as described above.

Imaging and Quantification

Images were obtained using a Leica confocal microscope, and Adobe Photoshop CS was used for image processing. Single optical z-sections of ~0.5 micron were used when appropriate to assess colocalization of markers. Quantification was performed by counting cells in 3 adjacent sections (per brain) through the rostral telencephalon, with cells in one z-section counted for each of the three 18 micron-thick sections. A total of at least 3 brains of the same age were analyzed for each experiment/condition. GraphPad Prism 5 software was used for significance tests.

In situ hybridization

For *in situ* hybridization, cDNA encoding zebrafish *vglut2a* was digested by NotI and transcribed using SP6 RNA polymerase and a digoxigenin (DIG) labeling mix to generate

antisense RNA probes. Adult zebrafish were anesthetized using tricaine and were perfused with PBS and 4% paraformaldehyde (Pfa). Fish were fixed in paraformaldehyde overnight and brains were dissected out the following day, embedded in 3% agarose, and processed for Vibratome sectioning (100 micron-thick) and subsequent *in situ* hybridization procedure as previously described (Adolf et al., 2006).

Transplantation of fezf2-GFP+ mutant cells into wildtype hosts

Transplantations were performed by taking 40 cells from 3-4hpf (hour post fertilization) *fezf2*-GFP/*too few* mutant zebrafish embryos and transplanting them into the animal pole of wildtype hosts of an equivalent stage. Hosts were screened for GFP expression at 24hpf (hours post fertilization), and corresponding donors were genotyped to identify whether they are heterozygous or homozygous mutants. Hosts were then grown to the adult stage (3 months old) for analysis.

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Concluding remarks

The work carried out in this dissertation establishes, for the first time, the expression of *fezf2* in neural progenitor/stem cells in the adult vertebrate telencephalon. In additional to labeling these cells, such a marker can be used to better understand the properties of adult neural progenitor cells, using lineage tracing approaches for example. And perhaps of even greater importance is the discovery of a molecular marker that is capable of regulating the expression of a number of other genes, as one would expect from a transcription factor.

Inspired by our discovery of *fezf2* expression within radial glial-like progenitor cells of the adult zebrafish telencephalon, we sought out to understand the functional role of this gene in the regulation of adult neural progenitor cells and adult neurogenesis. As discussed earlier, the adult zebrafish brain bears resemblance to the adult mammalian brain in certain aspects, one of which is the presence of proliferating cells within restricted germinal zones of the adult telencephalon. These telencephalic ventricular progenitor cells have a characteristic radial glial morphology, and are able to proliferate and give rise to new adult-born neurons. Again using the zebrafish as a vertebrate model system, we were able to investigate the function of this gene within adult neural progenitor cells.

Our findings suggest a role for *fezf2* in suppressing the proliferation of adult telencephalic neural progenitor cells. Analysis of the adult *too few* (Fezf2) mutant provides evidence that *fezf2* has an important role within the telencephalon, as we find that the adult *too few* homozygous mutant telencephalon is malformed and smaller compared to wildtype or heterozygote siblings. Analysis on sections leads us to the

observation of increased proliferation within the mutant ventricular zone, as well as an increase in adult neurogenesis. These findings identify a role for *fezf2* in suppressing the proliferation (and perhaps the subsequent differentiation) of telencephalic radial glial cells, further demonstrated through transplantation experiments in which transplanted heterozygous mutant cells seem to proliferate more than endogenous wildtype cells.

Taken together, our work establishes a role for *fezf2* in the regulation of neural progenitor cells within the adult zebrafish telencephalon, setting the stage for studies in mouse aimed at understanding whether *fezf2* plays a conserved role in mammals as well. Our study of the factors which regulate the proliferation and differentiation of neural progenitor cells is important both to better understand these processes at the cellular level, and also for the development of novel regenerative therapies that may one day hold promise in mammalian systems.

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