Published in final edited form as:

Gene Expr Patterns. 2009 September ; 9(6): 411–422. doi:10.1016/j.gep.2009.06.002.

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

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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.

Keywords

Fezf2; Radial glia; Ventricular progenitor cells; Telencephalon; Zebrafish; Transgenic; Adult neural stem cells; Adult neurogenesis; Olfactory bulb; Preoptic region; Hypothalamus

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.,

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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 (Becker and Becker, 2001; 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 known as *Fez1*, *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 stem 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 100 μ m 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).

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.

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.

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 (at the level of attachment to the telencephalon, see Figure 1A and Figure 2A), 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.

The zebrafish telencephalon is known to undergo eversion (Wullimann and Mueller, 2004). Though the olfactory bulb has partial pallial origin, it is largely non-everted. However, 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.

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)(the 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.

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. 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). 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.

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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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 Wullmann 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** supra commissural 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.

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 Wullmann et al., 1996. Abbreviations: **OB** olfactory bulb; **D** dorsal telencephalon; **V** ventral telencephalon; **VZ** ventricular zone; **Po** preoptic region; **Hc** caudal hypothalamus.

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 (VI region) (20X magnification). (H'-H''') Single confocal Z-section of the boxed region shows colocalization of *fezf2*-GFP with Hu in the VI region (40X magnification). Abbreviations: **D** dorsal telencephalon; **V** ventral telencephalon; **VZ** ventricular zone; **OB** olfactory bulb; **Vd** dorsal nucleus of V; **VI** lateral nucleus of V; **Vv** ventral nucleus of V.

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.

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.

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.

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 Wullmann 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.