Fezf2 Regulates Multilineage Neuronal Differentiation through Activating Basic Helix-Loop-Helix and Homeodomain Genes in the Zebrafish Ventral Forebrain

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Transcription factors of the *achaete-scute* and *atonal* bHLH proneural gene family play important roles in neuronal differentiation. They are also involved in neuronal subtype specification through collaboration with homeodomain (HD) transcription factors. However, concerted regulation of these genes and in turn progenitor fate toward distinct lineages within the developing vertebrate brain is not well understood. *Fezf2* is an evolutionarily conserved zinc finger protein important for monoaminergic neuronal development in zebrafish. Here, we show that *Fezf2* is also critical for GABAergic neuronal fate and investigate how a single transcription factor regulates the identity of multiple neuronal lineages in the developing ventral forebrain. First, our genetic analyses reveal the requirement of the *achaete-scute*-like genes *ascl1a* and *1b* in serotonergic and GABAergic neuron development, but they are dispensable for the specification of dopaminergic neurons, which is dependent on the *atonal*-like gene *neurog1*. Second, the expression of *fezf2*, *ascl1a/1b*, and *neurog1* demarcates distinct progenitor subpopulations, where *fezf2* is required for activating but not maintaining the expression of bHLH genes. Third, *Fezf2* is required to activate HD genes *otpb* and *dlx2*, which are involved in dopaminergic and GABAergic neuronal development, respectively. Finally, we uncover that *Fezf2* is sufficient to increase dopaminergic neuronal numbers but not serotonergic or GABAergic lineages. Together, these findings reveal new mechanisms by which multilineage differentiation is coordinately regulated by a single transcription factor in the vertebrate ventral forebrain.

Introduction

The development of multicellular organisms involves the production of diverse cell types that are of specific identity. The need for both diversity and specificity is particularly prominent in the vertebrate CNS, which initially unfolds from a simple monolayer of germinal neuroepithelia that have the potential to give rise to a vast repertoire of cell types (Jessell, 2000; Livesey and Cepko, 2001; Temple, 2001; Agathocleous and Harris, 2009; Kriegstein and Alvarez-Buylla, 2009). The generation of both proper numbers and types of differentiated cells over a prolonged period necessitates an orchestrated control of progenitor fate. Transcription factors encoded by the basic helix-loop-helix (bHLH)-containing proneural genes, originally discovered through classical genetic studies in *Drosophila* (Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1994), have been shown to play important roles in vertebrate neurogenesis (Anderson and Jan, 1997; Bertrand et al., 2002). Cell cycle exit and neurogenesis are

initiated in progenitor cells by upregulation of *atonal*-like *neurogenin* (Ma et al., 1996) and *achaete-scute*-like *ascl* genes (Guillemot et al., 1993). In addition to promoting the expression of neuronal differentiation markers, proneural genes are also critical for determining subtype-specific neuronal identity, through collaboration with transcription factors of the homeodomain (HD) class (Jessell, 2000; Shirasaki and Pfaff, 2002).

Fezf2 (also known as *Fezl*, ZNF312, or *Zfp312*) is an evolutionarily conserved forebrain transcription regulator (Shimizu and Hibi, 2009). In zebrafish embryos, *fezf2* is required for proper development of both dopaminergic (DA) and serotonergic (5-HT) neurons (Guo et al., 1999a; Levkowitz et al., 2003). The bHLH proneural gene *neurog1* is required for DA neuron development and is regulated by *Fezf2* (Jeong et al., 2006). However, it remains to be understood the nature of this regulation, and moreover, how *Fezf2* regulates the development of 5-HT neurons is an open question.

In this study, we uncover a new and essential role of *fezf2* in GABAergic neuron development and address how *fezf2* regulates the cell fate of multiple neuronal lineages in the developing zebrafish ventral forebrain. Our combined molecular genetic studies identify another family of bHLH genes, *ascl1a* and *ascl1b*, as essential regulators of 5-HT and GABAergic neuron development and show that *Fezf2* activates distinct bHLH genes in stereotypically positioned progenitor subpopulations. Additionally, *Fezf2* can also activate HD genes in these lineages, thereby directing them toward specific neuronal fate.

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Figure 1. GABAergic neurons marked by *gad67* transcript expression are reduced in the ventral forebrain of *tof^{m808}* mutant embryos (**A–D**) and *fezf2* morphants (**E–H**). The solid arrowheads point to the differences between wild-type, mutant embryos and *fezf2* morphants. Expression in the hindbrain was shown as controls (**C, D, G, H**). DT, Dorsal thalamus; H, hypothalamus; PT, posterior tuberculum; VT, ventral thalamus; Tel, telencephalon.

Materials and Methods

Zebrafish strains. Wild-type embryos (of either sex) were obtained from natural spawning of AB adults of both sexes and raised as described previously (Kimmel et al., 1995). The following mutants and transgenic lines (of either sex) were used: *tof^{m808}* (Guo et al., 1999a), *neurog1^{hi1059Tg}* (Golling et al., 2002), *pia^{t25214}* (Pogoda et al., 2006), *Tg(Hsp70::Gal4)* (Scheer and Camnos-Ortega, 1999), and *Tg(UAS::Fezf2)* (Jeong et al., 2007).

In situ hybridization, immunostaining, and 5-ethynyl-2'-deoxyuridine assay. RNA *in situ* hybridization and immunohistochemistry were performed as described previously (Guo et al., 1999b). For dual fluorescent *in situ* hybridization (FISH), the antisense RNA probe for *fezf2* was labeled with digoxigenin (DIG) (DIG RNA labeling mix; Roche), and the anti-DIG-peroxidase (POD) secondary antibody (Roche; 1:400) was used, followed by color development using Alexa Fluor 555 tyramide (Invitrogen). All other probes were labeled with dinitrophenyl (DNP) (DNP RNA labeling mix; NTP set from Fermentas; DNP-11-UTP from PerkinElmer), and the anti-DNP-POD secondary antibody (1:200; PerkinElmer) was used, followed by color development using Alexa Fluor 488 tyramide (Invitrogen). The procedure was performed as previously described (Filippi et al., 2007). For 5-ethynyl-2'-deoxyuridine (EdU) labeling, EdU was injected into the yolk of embryos at different developmental stages. Embryos were fixed at 48 h postfertilization (hpf) and stained according to the manufacturer's protocol (Invitrogen). For quantification of DA, 5-HT, or GABAergic neurons in the posterior tuberculum (PT)/hypothalamus (H) region, *in situ* or antibody-labeled cells were visualized under a compound differential interference contrast or fluorescent microscope, and counted manually. This was feasible because of the small number of these neu-

rons in the PT/H region. At least five embryos were quantified per genotype per condition.

Morpholino antisense oligonucleotide injection. Morpholinos (Gene Tools) were resuspended in nuclease-free water and stored at -80°C . The following gene-specific morpholinos were used in this study: *ascl1b* MO (Amoyel et al., 2005) and *fezf2* MO (Jeong et al., 2007); and 3–4 nl of 0.25 mM were used per embryo.

Results

Fezf2 is essential for the development of GABAergic neurons in the zebrafish ventral forebrain

We previously isolated the zebrafish mutant for *fezf2*, *too few* (*tof^{m808}*), which displays defects in the development of DA and 5-HT neurons (Guo et al., 1999a; Levkowitz et al., 2003) in the developing ventral forebrain, which is composed of the dorsal thalamus (DT), ventral thalamus (VT), PT, and H. To determine whether *fezf2* regulates the development of additional neuronal types, we examined GABAergic neurons using *in situ* hybridization for *Gad67*, a key enzyme involved in GABA synthesis. A loss of *gad67* expression was detected prominently in PT and H in the 48 hpf *tof^{m808}* mutant (Fig. 1B), whereas those in the telencephalon (Tel), DT, VT (Fig. 1B), and hindbrain (Fig. 1D) were largely unaffected. We have previously also developed morpholino antisense oligonucleotides targeting *fezf2*, and shown that *fezf2* morphants have a severer forebrain defect than the *tof^{m808}* mutant, such that the VT subdivision is defective (Jeong et al., 2007). Since VT is predominantly composed of GABAergic neurons, we examined *fezf2* morphants for their GABAergic neuronal state and found a significant deficit in VT (Fig. 1F), whereas those in the Tel, midbrain/hindbrain regions remained unaffected (Fig. 1F,H). Together, these results reveal that *fezf2* is essential for proper development of GABAergic neurons in the zebrafish ventral forebrain.

The development of DA versus 5-HT or GABAergic neurons depends on distinct bHLH proneural genes

How does *Fezf2* direct multilineage differentiation of neuronal progenitors toward DA, 5-HT, or GABAergic fate? Since our previous study uncovered an essential role of the proneural gene *neurog1* for DA neuron development (Jeong et al., 2006), we asked whether *neurog1* or other members of the proneural gene family are involved in 5-HT or GABAergic neuron specification, by analyzing these neuronal phenotypes in zebrafish mutants for *neurog1* (*neurog1^{hi1059}*) (Golling et al., 2002) and *ascl1a* (known as *pia^{t25215}*) (Pogoda et al., 2006). In agreement with the analysis of *neurog1* morphants (Jeong et al., 2006), DA neurons were reduced in the *neurog1^{-/-}* mutant (Fig. 2A,B,M), but 5-HT and GABAergic neurons were unaffected (Fig. 2C–F,M). After observing that all three neuronal types were normal in the *ascl1a/pia^{t25215}* mutant, we analyzed them in the *ascl1a/pia^{t25215}* mutant injected with a morpholino antisense oligonucleotide targeting

Figure 2. GABAergic, DA, and 5-HT neuron development in *neurog1*- or *ascl1a/b*-deficient embryos. DA, 5-HT, and GABAergic neurons in the control (**A, C, E, G, I, K**), *neurog1*^{-/-} mutant (**B, D, F, M**), and *ascl1b* morpholino-injected *ascl1a*^{-/-} mutant (*pia*^{t25215}) (**H, J, L, M**) embryos as revealed by whole-mount *in situ* hybridization with antisense RNA probes directed against *gad67*, *th*, or immunostaining with anti-5-HT antibody. DT, Dorsal thalamus; H, hypothalamus; PT, posterior tuberculum; VT, ventral thalamus; Tel, telencephalon. Please refer to Materials and Methods for quantification of these neurons in the PT/H region. Error bars indicate SEM. ***p < 0.001, **p < 0.01.

ascl1b and found that, while DA neurons were normal (Fig. 2G, H, M), 5-HT (Fig. 2I, J, M) and GABAergic (Fig. 2K–M) neurons in the PT and H were reduced. The hindbrain raphe 5-HT neurons were also reduced in *ascl1a* and *ascl1b* double-deficient embryos, consistent with a known role of *ascl1* family of genes in vertebrate raphe 5-HT neuronal development (Pattyn et al., 2004). Together, these results indicate that different bHLH genes differentially govern DA, 5-HT, and GABAergic neuronal development in the ventral forebrain.

The expression of *fezf2* and bHLH proneural genes implies regulatory hierarchy and defines distinct progenitor subpopulations

To discern the relationship between *fezf2* and proneural genes in neuronal specification, we analyzed their spatial and temporal expression patterns. Through whole-mount *in situ* hybridization, we found that *fezf2* was expressed in the forebrain primordium of the early gastrula embryo, preceding that of all three bHLH genes (Fig. 3A). As development progressed, proneural gene expression became detectable in subpopulations of forebrain progenitors (Fig. 3B, C). Dual FISH at ~24 hpf provided a higher resolution view of their expression patterns through confocal microscopy. Surprisingly, only a small population of *fezf2*⁺*neurog1*⁺ cells was detected in the central domain of VT, among those that are singly positive for either *fezf2* or *neurog1* (Fig. 3D). An even smaller overlap was detected between the expression of *fezf2* and *ascl1a* (Fig. 3E) or *ascl1b* (Fig. 3F), in anterior H and dorsal VT, respectively. These results reveal distinct progenitor subpopulations in the ventral forebrain demarcated by their expression profiles of *fezf2* and bHLH genes.

Fezf2 is required to activate bHLH genes in distinct progenitor subpopulations

As previously reported (Jeong et al., 2006), we observed a transient but highly reproducible reduction of *neurog1* expression in the ventral forebrain (Fig. 4B) of the ~26-somite stage (~22 hpf) *tof*^{m808} mutant, and this reduction appeared exacerbated in the *fezf2* morphant (Fig. 4D). Similar reduction of gene expression was observed for *ascl1a* (Fig. 4E–H). Unexpectedly, however, *ascl1b* expression was slightly increased in the ventral forebrain of the *tof*^{m808} mutant (Fig. 4I, J) but appeared reduced in the *fezf2* morphant (Fig. 4K, L). This increased *ascl1b* expression could be due to the loss of *neurog1* in the *tof*^{m808} mutant, as *neurog1* was previously shown to repress *ascl1* expression in the mouse brain (Fode et al., 2000). We examined *ascl1b* expression in the *neurog1*^{-/-} mutant, and indeed an increased *ascl1b* expression was observed in the ventral forebrain of the *neurog1*^{-/-} mutant (Fig. 5), supporting the notion that increased *ascl1b* expression in the *tof*^{m808} mutant may be indirectly due to the loss of *neurog1*-mediated repression, whereas a further reduction of *fezf2* activity in the morphant uncovers Fezf2-dependent activation of *ascl1b*. Together, these results provide genetic evidence to support that Fezf2 is an upstream activator of bHLH genes in the ventral forebrain.

Fezf2 is required to activate the HD genes *otpb* and *dlx2* in distinct ventral forebrain progenitor subpopulations

The bHLH proneural genes specify neuronal subtype identity through collaboration with HD genes (Scardigli et al., 2001; Lee and Pfaff, 2003). We therefore examined whether Fezf2 regulated HD genes in addition to proneural genes. The HD gene *otpb* is expressed in the ventral forebrain (Fig. 6A) and is required for the

Figure 3. Expression patterns of *fezf2* and proneuronal genes. **A–C**, *fezf2* expression precedes that of bHLH genes during early development. Images of whole-mount ISH with antisense RNA probes directed against *fezf2*, *neurog1*, *ascl1a*, *ascl1b* in wild-type embryos at 8 hpf (**A**), 12 hpf (**B**), and 14 hpf (**C**). **D–F**, Lateral views ($\sim 5 \mu\text{m}$ confocal projection, which is smaller than an average cell diameter of $\sim 10 \mu\text{m}$) of the 20 hpf embryonic brains show the expression of *neurog1* (**D**), *ascl1a/b* (**E, F**) (green), and *fezf2* (red), revealed by FISH. H, Hypothalamus; Dien, diencephalon; PT, prethalamus; VT, ventral thalamus; Tel, telencephalon.

Figure 4. Proneuronal gene expression in *fezf2*-deficient embryos. Images of whole-mount *in situ* hybridization with antisense RNA probes directed against *neurog1*, *ascl1a*, and *ascl1b* in wild-type (**A, E, I**), *tof*^{m808} mutant embryos (**B, F, J**), control morpholino- (**C, G, K**), and *fezf2* morpholino-injected embryos (**D, H, L**) at ~ 20 hpf. All images are lateral views of anterior brain regions. Anterior is to the left, and dorsal is up. H, Hypothalamus; PT, posterior tuberculum; Tel, telencephalon.

specification of DA neurons (Blechman et al., 2007; Ryu et al., 2007). Another HD gene, *dlx2*, which is known to regulate GABAergic neuronal development in mammals (Anderson et al., 1997), is also expressed in the region (Fig. 6B). In the *fezf2* mutant and morphant, the expression of these genes was defective in distinct subpopulations of progenitors (Fig. 6C–J). Thus, these results suggest that Fezf2 can activate HD genes in addition to bHLH genes.

Fezf2 is sufficient to increase DA but not 5-HT and GABAergic neurons

Given that *fezf2* is required for the development of DA, 5-HT, and GABAergic neurons, we next asked whether it is sufficient to generate more of these neurons when overexpressed. Since direct delivery of *fezf2* mRNA into one-cell stage embryos causes severe deformity, we examined the consequence of *fezf2* overexpression using the heat shock promoter and *Gal4-UAS* system (Scheer and Camnos-Ortega, 1999; Jeong et al., 2007). To determine when is the appropriate time for heat shock, we performed *in vivo* birth-dating analysis for the small and quantifiable numbers of DA, 5-HT, and GABAergic neurons in the PT/H region, which were also most severely affected in the *to^{pm808}* mutant (Fig. 7). By injecting EdU into wild-type embryos at different developmental stages and analyzing cells that are double-labeled with EdU and neuron-specific markers at 2 d postfertilization (dpf) (Fig. 7A), we found that DA, 5-HT, and GABAergic neurons in PT/H were born in a highly intermingled fashion, with >60% being generated during a common developmental window from 10 to 18 hpf (Fig. 7B–F).

We therefore performed heat shock at multiple developmental stages from 6-somite to 14-somite, and analyzed the state of DA, 5-HT, and GABAergic neurons at 48 hpf. Induction of *fezf2* at 6-, 8-, and 10-somite stages led to significantly increased production of DA neurons in the ventral forebrain, and some increase of hindbrain locus ceruleus noradrenergic neurons were also observed (Fig. 8A,D). *fezf2* overexpression, however, did not increase GABAergic or 5-HT neurons at any stages during which heat shock was performed (Fig. 8B–D).

To further explore how *fezf2* overexpression increases DA but not 5-HT or GABAergic neurons, we asked whether *fezf2* is sufficient to differentially activate proneural and HD genes. Heat shock was performed at the 10-somite stage, and embryos were processed for *in situ* hybridization at 20 hpf. *Fezf2* overexpression was sufficient to increase the expression of *neurog1* (Fig. 8E,F) and *otpb* (Fig. 8G,H), but not that of *ascl1a* (Fig. 8I,J), *ascl1b* (Fig. 8K,L), or *dlx2* (Fig. 8M,N). It is worth pointing out that the increase in *neurog1* and *otpb* expression appeared as an expansion of the endogenous expression domains rather than a formation of new domains at ectopic locations. These results suggest that Fezf2 is a rate-limiting factor in the induction of *neurog1* and *otpb* expression in the ventral forebrain.

Discussion

How multipotent progenitors give rise to multiple neuronal lineages during brain development is not well understood. Through combined molecular genetic loss- and gain-of-function approaches, we show that Fezf2 guides ventral forebrain neural progenitors toward DA, 5-HT, or GABAergic neuronal fate through activating *achaete-scute* and *atonal* type of bHLH

Figure 5. Ectopic *ascl1b* expression in *neurog1*-deficient embryos. The arrowhead indicates the increased expression in the dorsal telencephalon; the arrows indicate the increased *ascl1b* expression in the diencephalic region. All images are lateral views of anterior brain regions. Anterior is to the left, and dorsal is up.

proneural as well as HD genes in stereotypically positioned progenitor subpopulations (Fig. 8O, a schematic model). This finding provides new insights into the government of multilineage differentiation of neuronal progenitor cells, with potential implications for improving specific reprogramming of pluripotent stem or somatic cells into appropriate neuronal lineages.

Our findings also prompt a new question: How does Fezf2 activate different bHLH genes in distinct subsets of progenitors? The highly stereotyped patterns of organization of DA, 5-HT, and GABAergic progenitor domains and neurons in the ventral forebrain argue against a model of stochastic induction. Thus, *fezf2*-expressing ventral forebrain progenitors must represent a heterogeneous population of cells, either due to extrinsic or intrinsic programming. Future studies at single-cell resolution and at biochemical levels are necessary to further parse out the complex gene regulatory network underlying multilineage differentiation.

Does Fezf2 directly activate proneural genes in the zebrafish ventral forebrain? It is worth noting that Fezf2 contains a conserved engrailed homology 1 (EH1) repressor motif. The EH1 type of motif is known to interact with Groucho or Transducin-like enhancer of split (TLE) type transcriptional corepressors (Shimizu and Hibi, 2009). A study in the mouse dorsal telencephalon shows that Fezf2 together with its paralog Fezf1 directly represses the expression of *Hes5*, thereby indirectly activating Neurogenin 2 (Shimizu et al., 2010). Interestingly, however, many Groucho or TLE-interacting proteins, for example, TCF/LEF family of proteins involved in the Wnt signaling pathway (Bienz, 1998), are known to function as both activators and repressors, depending on intramolecular and intermolecular interactions in distinct cellular contexts. Thus, it remains a possibility that Fezf2 can directly activate proneural genes in the zebrafish ventral forebrain. Indeed, we have observed that Fezf2 appears to have distinct functions in the zebrafish dorsal Tel versus in the ventral forebrain (N. Yang, unpublished data). It would therefore be of interest in the future to analyze the role of Fezf2 in zebrafish telencephalic neurogenesis, as well as determining the biochemical nature of Fezf2 regulation of proneural genes.

Expression analyses as well as genetic studies support a priming rather than a maintenance role of Fezf2 in regulating the expression of bHLH and HD genes. First, at the mRNA level, a rather minimal overlap between the expression of *fezf2* and bHLH or HD genes is detected, suggesting that *fezf2* expression is downregulated shortly after the induction of bHLH and HD genes in a temporally specific manner. Second, impairment of *fezf2* activity reduces rather than abolishes bHLH and HD gene expression. While incomplete inactivation of *fezf2* is a possible

Figure 6. Fezf2 regulates the expression of HD genes *otpb* and *dlx2*. **A, B**, Small subsets of *fezf2* + progenitor cells express *otpb* or *dlx2* as revealed by FISH. Lateral views of the 20 hpf embryonic brains show that the expression of *otpb* (**A**), *dlx2* (**B**) (green) displays small overlap with that of *fezf2* (red). **C–J**, Images of whole-mount *in situ* hybridization with antisense RNA probes directed against *otpb* and *dlx2* in wild-type (**C, G**), *tof m808* mutant embryos (**D, H**), control morpholino– (**E, I**), and *fezf2* morpholino-injected embryos (**F, J**) at ~20 hpf. All images are lateral views of anterior brain regions. Anterior is to the left, and dorsal is up. DT, Dorsal thalamus; H, hypothalamus; VT, ventral thalamus; Tel, telencephalon.

cause, the data also suggest that other factors in the region may be able to activate these genes in the absence of *fezf2*. Consistent with this idea, the loss of bHLH gene expression in the ventral forebrain is only transiently detectable in ~22 hpf *fezf2*-deficient embryos. Nevertheless, such defect has detrimental effects on neuronal lineage differentiation. Together, we propose that *fezf2* plays a temporally critical role in promoting the differentiation of neuronal progenitors into early-born neurons, through priming but not maintaining the activation of bHLH and HD genes.

Our gain-of-function experiments reveal that overexpression of Fezf2 is sufficient to increase DA but not 5-HT or GABAergic neurons in the ventral forebrain. This is in part due to the ability of Fezf2 to increase the expression of *neurog1* and *otpb* but not that of *ascl1a*, *1b*, and *dlx2*. These results reveal a rate-limiting effect of Fezf2 in DA neuron development and suggest that Fezf2 exerts a different mode of regulation in different neuronal lineages. What accounts for this difference is not clear. One possibility is that *fezf2* may be expressed at different levels in distinct progenitor populations, thereby exerting a concentration-dependent effect on their differentiation. It will be of interest to examine whether and how *fezf2* may be differentially expressed in progenitor cells, and what impact it might have on progenitor fate choice.

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Figure 7. Birth-dating analysis of DA, 5-HT, and GABAergic neurons in the ventral forebrain. **A**, EdU was injected at different embryonic stages as indicated. At 48 hpf, embryos were collected and subjected to EdU staining together with immunofluorescence or FISH to mark specific neuronal subtypes. **B**, Schematic representations of the examined neuronal types in a 2 dpf wild-type embryo. **C–E**, Single confocal Z section ($\sim 1 \mu\text{m}$ thick) of wild-type zebrafish embryos at 48 hpf. Proliferating DA (**C**), 5-HT (**D**), and GABAergic (**E**) progenitors were labeled by EdU at 14 hpf, and visualized by EdU staining together with immunofluorescence with antibodies against tyrosine hydroxylase (TH) and 5-HT or whole-mount FISH with antisense RNA probe directed against *gad67*. The arrows indicate EdU⁺ neurons, whereas the arrowheads indicate neurons with no EdU staining. **F**, Plot of the percentage of proliferating DA, 5-HT, and GABAergic progenitor cells as a function of time in wild-type embryos ($n = 3–5$ for individual neuronal type), showing that they are born in a similar developmental window. All images are lateral views of anterior brain regions. Anterior is to the left, and dorsal is up. Please refer to Materials and Methods for quantification of these neurons in the PT/H region.

Figure 8. Neurogenesis in the ventral forebrain of *fezf2*-overexpressing embryos. DA (**A**), GABAergic (**B**), and 5-HT (**C**) neurons in embryos with forced *fezf2* expression at different stages during embryonic development. **D**, Quantifications of DA, GABAergic, and 5-HT neurons in forebrain and hindbrain region of nontransgenic control and *fezf2* overexpressing embryos. $n \geq 5$ per genotype. *** $p < 0.001$; ** $p < 0.01$. Error bars indicate SEM. **E–N**, Embryos derived from a cross between *Hsp70::Gal4* and *UAS::Fezf2* transgenic fish are heat shocked at 14 hpf and stained with *neurog1* (**E**, **F**), *otpb* (**G**, **H**), *ascl1a* (**I**, **J**), *ascl1b* (**K**, **L**), and *dlx2* (**M**, **N**)-directed probes at 20 hpf. **O**, A schematic model. Fezf2 regulates the generation of DA neurons through activating *neurog1* and *otpb*. Meanwhile, Fezf2 regulates the production of 5-HT and GABAergic neurons through activating *ascl1a/1b*, *dlx2*, respectively, and possibly other undetermined mechanisms. DT, Dorsal thalamus; H, hypothalamus; PT, posterior tuberculum; VT, ventral thalamus; Tel, telencephalon. Please refer to Materials and Methods for quantification of these neurons in the PT/H region.

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