Transient Expression of Fez Family Zinc Finger 2 Protein Regulates the Brn3b Gene in Developing Retinal Ganglion Cells*

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Chunsheng Qu1,‡,1, Dandan Bian§,1, Xue Li¶,1, Jian Xiao¶,1, Chunping Wu§,1, Yue Li**, Tian Jiang§,1, Xiangtian Zhou§,1, Jia Qu†,1, and Jie-Guang Chen‡,1

From the 1School of Ophthalmology and Optometry and Eye Hospital, Wenzhou Medical University, Wenzhou, Zhejiang 325000, China, the 2China State Key Laboratory Cultivation Base and Key Laboratory of Vision Science, Ministry of Health of China, Wenzhou, Zhejiang 325000, China, the 3Zhejiang Provincial Key Laboratory of Ophthalmology and Optometry, Wenzhou, Zhejiang 325000, China, the 4Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, Connecticut 06520, and the 5Clinical Laboratory of LiShui People’s Hospital, Sixth Affiliated Hospital, Wenzhou Medical University, LiShui, Zhejiang 323000, China

Retinal ganglion cells (RGCs) are projection neurons in the neural retina that relay visual information from the environment to the central nervous system. The early expression of MATH5 endows the post-mitotic progenitors with RGC competence and leads to the activation of Brn3b that marks committed RGCs. Nevertheless, this fate commitment process and, specifically, regulation of Brn3b remain elusive. To explore the molecular mechanisms underlying RGC generation in the mouse retina, we analyzed the expression and function of Fez family zinc finger 2 (FEZF2), a transcription factor critical for the development of projection neurons in the cerebral cortex. Fezf2 mRNA and protein were transiently expressed at embryonic day 16.5 in the inner neuroblast layer and the prospective ganglion cell layer of the retina, respectively. Knockout of Fezf2 in the developing retina reduced BRN3B+ cells and increased apoptotic cell markers. Fezf2 knockdown by retinal in utero electroporation diminished BRN3B but not the coexpressed ISLET1 and BRN3A, indicating that the BRN3B decrease was the cause, not the result, of the overall reduction of BRN3B+ RGCs in the Fezf2 knockout retina. Moreover, the mRNA and promoter activity of Brn3b were increased in vitro by FEZF2, which bound to a 5′ regulatory fragment in the Brn3b genomic locus. These results indicate that transient expression of Fezf2 in the retina modulates the transcription of Brn3b and the survival of RGCs. This study improves our understanding of the transcriptional cascade required for the specification of RGCs and provides novel insights into the molecular basis of retinal development.

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† Both authors contributed equally to this work.

‡ To whom correspondence should be addressed: Key Laboratory of Visual Science and School of Optometry and Ophthalmology, Wenzhou Medical University, 270 Xueyuan Rd., Wenzhou, Zhejiang 325003, China. Tel.: 86-577-88067927; Fax: 86-577-88067934; E-mail: jgchen@mail.eye.ac.cn or jiesbooks@gmail.com.

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been extensively studied by in vivo genetic manipulations. It was found that transcription factor FEZF2 (also known as FEZL and ZFP312) was expressed by early neural progenitors in the ventricular zone of the dorsal telencephalon. Zinc finger protein FEZF2 represses the expression of Hes5 in proliferating neural stem cells (15, 16) and regulates the differentiation of telencephalic precursors from mouse embryonic stem cells (17). Fezf2 is required for the proper specification and differentiation of subcerebral projection neurons in the deep layers of the cerebral cortex (18–20). Overall, these observations led us to hypothesize that Fezf2 may regulate the specification of retinal projection neuron RGCs.

In this study, we examined the expression and function of Fezf2 in developing retinas. We found that Fezf2 was transiently expressed in retinas during the generation of RGCs. To investigate the role of Fezf2, we inhibited its expression by RNA interference and deleted the gene by conditional Fezf2 knockout in the retina. Our results showed that knockdown and knockout of Fezf2 led to BRN3B inhibition and impaired the formation of RGCs. Moreover, Fezf2 regulated the transcription of Brn3b by binding to a 5′ regulatory sequence of the Brn3b locus.

Experimental Procedures

Animals and Genotyping—Animal procedures were performed according to the protocol approved by the Animal Care Committee of Wenzhou Medical University. Tg (Fezf2-EGFP) mice were created by the GENSAT project and obtained from the Mutant Mouse Regional Resource Center. Fezf2fl/fl mice were provided by Nenad Sestan (Yale University). Exon 2, which encodes amino acids 1–279 of 455 in FEZF2, was targeted for removal (21). The Tg(Six3-cre)69Frty mouse was created by Yasuhide Furuta (RIKEN Center for Developmental Biology) (22) and was initially obtained from Lin Gan (University of Rochester Medical Center). Dr. Furuta granted permission for its use by our group. The Fezf2 second exon was deleted in retinas by crossing Fezf2fl/fl mice with Six3-Cre mice. The retinal-specific knockout of Fezf2 was identified by PCR genotyping using three primers located in exon 1–3 of Fezf2 as described before (21). The loss of Fezf2 expression was further confirmed by quantitative PCR (q-PCR) or Western blotting in one eye, whereas the counterpart eye from the same embryo was taken for the knockout study.

Tissue Preparation—Mouse pups were put on ice to induce hypothermia while pregnant dams were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Eyes from prenatal mice were enucleated under a dissecting microscope and fixed by immersion in 4% paraformaldehyde. All tissues were cryoprotected in 30% sucrose at 4 °C, frozen by liquid nitrogen vapor, and then embedded in OCT compound (Sakura Tissue). The sections were incubated overnight at 4 °C with primary antibodies diluted in blocking solution. The antibodies used in this study were mouse anti-SMIB32 (1:200, Covance, 14942601), goat anti-BRN3B (1:50, Santa Cruz Biotechnology, sc-31989), rabbit anti-MATH5 (1:200, Abcam, ab78046), rabbit anti-ISLET1 (1:200, Abcam, ab20670), rabbit anti-caspase III (1:200, Cell Signaling Technology, 9622), goat anti-BRNN3A (1:50, Santa Cruz Biotechnology, sc-31984), and rabbit anti-FEZF2 (1:500, Abcam, ab69436). The samples were processed with secondary antibodies: Cy3-conjugated anti-rabbit IgG and dylightTM549-conjugated anti-goat IgG (Jackson Immunoresearch Laboratories) at a 1:400 dilution. Fluorescence images were taken using a laser-scanning confocal microscope (Zeiss).

RNA in Situ Hybridization—Nonradioactive RNA in situ hybridizations of Fezf2 were performed as described previously (18). The digoxigenin-labeled Fezf2 riboprobe (corresponding to nucleotides 1241–1734 of NM_080433) was generated by in vitro transcription from a linearized TopoII-Fezf2 plasmid. The cryosections were hybridized with the probe in hybridization buffer (50% formamide, 4× SCC, 50 μM NaH2PO4, 100 μg/ml tRNA, 7.5% dextran sulfate, 1× Denhardt’s solution, and 500 μg of salmon sperm DNA) overnight at 65 °C. After high-stringency post-hybridization washes, the hybridization signal was detected with an alkaline phosphatase-conjugated anti-digoxigenin antibody and developed using nitro blue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate chromogen (Roche). Hybridization pictures were taken with a Nikon microscope (SMZ1500) and an Olympus microscope (EX41).

Plasmid Constructs—For in vivo inhibition of Fezf2 expression, we used Zfp312 siRNA-1 and Zfp312 siRNA-2 with targeting sequences GGAAACTCGCCCTTGGCA at 775–793 and GGTGTCTAATGCTCAGAT at 886–904 of the coding region of Fezf2, respectively. The scrambled scRNA-1 and scRNA-2 were created by introducing three mutations in the target sequences. The Zfp312 siRNA-1 and its scramble were cloned in the lentiviral vector pLVTH, whereas Zfp312 siRNA-2 and its scramble were created on the basis of pGLH. All constructs were verified in a previous study of Fezf2 functions in the cerebral cortex (18). For Fezf2 expression, full-length Fezf2 was cloned into pCAGEN (Addgene), which harbors a CAG promoter. For ChIP, a V5 tag (GGPnPNNLGLDST) was inserted into Fezf2-pCAGEN and pCAGGS-GFP following the startup codon ATG of Fezf2 and GFP, respectively, by PCR in-frame cloning.

To measure the promoter activity in the 5′ regulatory region of Brn3b, a conservative 1-kb fragment upstream from the starting site of Brn3b was amplified by PCR using primers tcgtctttcttcctttgaa and cggcaataatctaataatagctgtcaa. The PCR product was cloned into pGL4.18, a promoterless firefly luciferase vector co-expressing a neomycin resistance gene (Pro-mega). Mouse neuroblastoma N2a cells were transfected with the pGL4.18 promoter and cultured in the presence of G418 for 2 months to select cells stably expressing the plasmid. All constructs were confirmed by sequencing prior to the in vivo and in vitro experiments.

Retinal in Utero Electroporation—Plasmids were delivered to the developing retina by retinal in utero electroporation (23).
Briefly, E13.5 pregnant mice were anesthetized and attached to a clean table. The abdominal cavity was opened to expose the uterine horns. DNA solution (4 µg/µl in Tris-EDTA buffer containing 0.1% fast green) were injected through the uterus wall using a polished pulled glass micropipette. The head of each embryo was placed between tweezer-type electrodes, and five square electric pulses (38 V, 50 ms) were passed at 1-s intervals using a BTX electroporator. The wall and skin of the abdominal cavity were aligned and sutured. The embryos were allowed to develop in vivo until E17.5 or postnatal day 7 (P7). Then the mice were sacrificed to remove the brains together with the eyes for fixation in paraformaldehyde.

**FACS**—To study the effect of Fezf2, we isolated E17.5 retinas from Fezf2-siRNA-transfected mice. Retinas were peeled from the eyeballs and collected under a stereomicroscope (Zeiss). The tissues were minced and incubated with 0.05% trypsin for 8 min at 37 °C. Single cell suspensions were prepared by trituration, suspension, and filtration. GFP- cells were separated by FACS using a FACS Aria II cell sorter (BD Biosciences) and were taken for measuring gene expression by q-PCR.

**Real-time RT-PCR**—To quantitate the gene expression of Fezf2 and Brn3b, we synthesized cDNA from the RNA isolated from the retina (SuperScript™ first-strand synthesis system, Invitrogen). q-PCR was performed using an ABI 7900 system (Applied Biosystems) and monitored on the basis of SYBR Green I dye detection with the primer sets for Fezf2 (accaggtgccaggaag and tcggaacgcatctccttg) and Brn3b (tcgctttcttcccttgaa and cggcaataaatataatagctgtcaa). The primer sets are unique to the targeted genes and do not map to any other genes in the mouse genome according to a BLAST search. Gene expression levels were normalized to the reference gene Actin. The Ct value was defined as the number of PCR cycles required for the fluorescent signal to grow beyond a threshold.

**Chromatin immunoprecipitation**—ChIP was performed by following the protocol of the EZ ChIP kit (Millipore, 17-371). N2a cells were transfected with pCAG-V5-Fezf2 and pCAG-V5-GFP. The cells were cross-linked with 1% paraformaldehyde and lysed on ice. The lysates were sonicated to shear the chromatin and incubated overnight at 4 °C with anti-V5 antibody bound to Dynal protein A/G beads (1:10, Abcam, ChIP grade ab9116). The precipitate from V5-Fezf2 and V5-GFP in Dynabeads was extensively washed and eluted at 65 °C for 15 min with elution buffer (1% SDS and 10% 1 M NaHCO3 in H2O). The eluted protein-chromatin complexes were reverse-cross-linked by incubation with 5 M NaCl overnight at 65 °C. Both ChIPed and input DNA were treated with RNaseA and proteinase K, followed by DNA purification.

**PCR and q-PCR of ChIP DNA**—To determine whether FEZF2 binds to Brn3b genomic DNA, we amplified a tentative regulatory region of Brn3b (−315−+310) (chr8, 78436342–78436967) by PCR with a primer set (GTGCAAGCTCGTCCCTGATAGC and ATCAGGAGTGGCCTGCTG). The input DNA from the transfected cells was taken as a positive control for gel analysis of ChIP DNA. In a similar manner, the region covering FEZF2 binding motifs (CAGCAACC) (−230−−137) was examined by q-PCR with primers (GTAAGCTCGCCCTGTAGC and GAGGCGAGCCCTGC). A part of the 3′ UTR of Brn3b (1507−1595) was amplified as a negative control by primers (ACCGGAATTGTTTTCTGATAGC and CTTTCCCTTCCCTCTT).

**Data Analysis**—For all q-PCRs and the luciferase assay, data were presented as mean ± S.D. from three independent experiments with duplication of reading. For cell counting from immunostaining, mean ± S.D. were calculated from 12 sections on the basis of three to four samples (eyes). The control and experimental groups were compared by independent samples t test using SPSS 17.0 software (SPSS Inc.) (*, p < 0.05; **, p < 0.005; ***, p < 0.0005).

**Results**

**Transient Expression of Fezf2 in the Developing Retina**—Retinal cells are generated consecutively from neuroepithelium in the optic cup starting from E11.5 and continuing into the early postnatal period (24). RGCs are the first born retinal cells generated from mid- to late gestation (24, 25). We measured the expression of Fezf2 in the retina by RNA in situ hybridization. Fezf2 signals were not detected in the E12.5 retina but appeared by E14.5 and E16.5, when massive RGC production occurred (25). Expression of Fezf2 was diminished by P1 and disappeared at P7 (Fig. 1A), when RGC generation was completed. Fezf2 mRNA was expressed in the inner neuroblast layer (NBL) of the retina but not detected clearly in the outer neuroblast layer, a presumptive ganglion cell layer (GCL) (Fig. 1B). Compared with the neocortex, the retina had a relatively weaker expression of Fezf2 (Fig. 1C). The temporal expression pattern of Fezf2 suggests that this transcription factor may be involved in regulating RGC development.

Although Fezf2 mRNA was hardly detected in the GCL of the developing retina when assayed by in situ hybridization, the FEZF2 protein was found in the GCL at E16.5, as revealed by IF (Fig. 2A). FEZF2 was co-localized with BrN3B (Fig. 2B), a marker protein of RGCs, indicating FEZF2 expression in the developing RGCs. The discrepancy in the location of Fezf2 mRNA and protein is likely caused by the transient expression...
in the migrating neurons. FEzf2 protein may start to be transcribed from the transiently expressed gene in the NBL and, with a possibly long half-life, accumulate in post-migratory neurons in the GCL. The presence of FEzf2 in the GCL allows the protein to participate in regulating the development of post-mitotic neurons in the retina. The Fezf2 expression pattern is akin to that of Math5. A knockin lacZ at the Math5 locus was expressed throughout the retina, including the GCL, despite Math5 mRNA appearing only in the NBL at E15.5 (11).

Fezf2 Knockout Reduces RGCs and Increases Apoptotic Cells—To assess the role of Fezf2 in RGC development, we analyzed Fezf2-null retinas generated by crossing Fezf2<sup>flox/flox</sup> and Six-3-Cre mice. Fezf2 deletion was confirmed by the presence of the inserted loxp and absence of the Fezf2 second exon, as determined by genotyping PCR (Fig. 3, A and B). As expected, expression of Fezf2 decreased in the isolated null retina compared with the control on E17.5 (Fig. 3C). Knocking out Fezf2 attenuated Brn3b mRNA levels (Fig. 3C) and reduced BRN3B protein in the isolated retina (Fig. 3D). The number of BRN3B+ cells was reduced in the retinal sections from knockout mice (Fig. 3E). The wild-type retina had 42.6 ± 3.0 BRN3B+ cells (per field, ×40) in the GCL, which decreased to 23.8 ± 2.1 when Fezf2 was deleted (Fig. 3F). We also noticed an almost complete reduction of BRN3B+ cells in the NBL (Fig. 3, E and F). A few BRN3B+ cells scattered in the NBL of wild-type mice represent the newly generated ganglion cells en route to the GCL.

Previous studies have established that Brn3b is required for preventing nascent RGCs from apoptotic cell death during retinal development (5, 26). Therefore, we investigated apoptotic cells in control and Fezf2-null retinas by the activation of CASPASE III. The cells with activated CASPASE III were found in development (5, 26). Therefore, we investigated apoptotic cells in control and Fezf2-null retinas by the activation of CASPASE III. The cells with activated CASPASE III were found in the GCL of the Fezf2<sup>−/−</sup> retina (Fig. 3E). The Fezf2-deleted retina had 2.5-fold more CASPASE III+ cells than the wild type at E17.5 (Fig. 3G), when maximum apoptosis occurs in Brn3b knockout mice (5). The number of apoptotic cells in the Fezf2 knockout retina is comparable with that in the Brn3b-deleted retina at E17.5 (5). The increase in apoptosis in the Fezf2<sup>−/−</sup> retina suggests that Fezf2 is needed for the proper function of Brn3b in the developing retina.

The reduction of RGC marker BRN3B in the developing retina at E17.5 (5). The increase in apoptosis in the knockout retina is comparable with that in the Brn3b-deleted retina at E17.5 (5). The increase in apoptosis in the Fezf2<sup>−/−</sup> retina suggests that Fezf2 is needed for the proper function of Brn3b in the developing retina.

Types that reduced in Fezf2<sup>−/−</sup> retinas, we immunostained retinas with anti-SMI-32, which predominantly labels the axons of RGCs (6). As shown in Fig. 4C, Fezf2<sup>−/−</sup> retinas had a decreased population of SMI-32 immunoreactive processes, suggesting a loss of RGC axons. Consistently, the optic nerve appeared thinner in the Fezf2-deleted retina compared with the control (Fig. 4D). In addition, we noticed that axons from Fezf2 knockout RGCs occasionally bifurcated within the retina (Fig. 4C), reminiscent of aberrant axonal trajectories as seen in the Brn3b knockout retina (27). All of these results support the hypothesis that the transient expression of Fezf2 is essential for the proper expression of Brn3b and formation of RGCs.
Fezf2 Knockdown Inhibits BRN3B Expression—Fezf2 knockdown reduced BRN3B+ RGCs in the retina, either as direct effects of inhibition of Brn3b expression or as effects of developmental changes leading to loss of BRN3B+ RGCs indirectly. To determine whether the loss of BRN3B+ RGCs was caused by a cell-autonomous or a non-cell-autonomous mechanism, we knocked down Fezf2 in a small population of retinal cells within the context of a healthy environment by retinal in utero electroporation. Fezf2 in the developing retina was knocked down with lentiviral shRNA that specifically inhibits Fezf2 expression by RNA interference (18). Plasmid pLVTH-Fezf2-siRNA-1, which harbors a reporter gene, GFP, was delivered into the eye cups of E13.5 embryos by in utero electroporation. Fezf2 inactivation knocked down with lentiviral shRNA that specifically inhibits Fezf2 expression by RNA interference (18). Plasmid pLVTH-Fezf2-siRNA-1, which harbors a reporter gene, GFP, was delivered into the eye cups of E13.5 embryos by in utero electroporation. Fezf2 knockdown abolished BRN3B+ cells, but not ISLET1, which coexpresses with BRN3B, demonstrating that Fezf2 is essential for the expression of Brn3b rather than controlling the survival of BRN3B+ RGCs directly.

In mature retinas, BRN3A and BRN3B are each expressed in approximately 70% of RGCs in an overlapping pattern. Brn3a was considered a downstream target of Brn3b (28). To evaluate the expression of BRN3A in the cells with diminished BRN3B, we knocked down Fezf2 with Fezf2-siRNA-2. Fezf2 inhibition did not significantly change the ratio of colocalization of BRN3A+ and the transfectants (Fig. 5, D and E), indicating that Fezf2 inhibition had no effect on the expression of BRN3A. Our result is consistent with a previous study with a knockin alkaline phosphatase to visualize individual genetically altered RGCs. It was found that Brn3b is not required for the maintenance of Brn3a expression in RGCs. The loss of Brn3a in Brn3b-null retinas is more likely due to an overall reduction in BRN3A+ RGC number rather than inhibition of Brn3a expression (27).

Because FEZF2 is a transcription factor, we envisioned that the transcription and mRNA levels of Brn3b may be subject to regulation by FEZF2. To test this, we inhibited Fezf2 expression by electroporating the pLVTH-Fezf2-siRNA-1 and pLVTH-Fezf2-siRNA-1 plasmids into the eye cups of E13.5 embryos.
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FIGURE 6. Overexpression of Fezf2 alters the phenotype of retinal cells. A, retinas transfected with pCAGEN and Fezf2-pCAGEN were examined on E17.5 with IF. Colocalization of MATH5, ISLET1, and BRN3B (red) with GFP was determined by IF. B, the percentages of colocalization in the pCAGEN and Fezf2-pCAGEN groups. Overexpression of Fezf2 abolished the expression of MATH5, ISLET1, and BRN3B. Scale bars = 200 μm and 20 μm (insets). **, p < 0.005; ***, p < 0.0005.

The electroporated cells were dissociated from the retinas at E17.5 and isolated by FACS on the basis of expression of the reporter GFP. Assays with q-PCR revealed that Fezf2-siRNA expressing cells had a decreased level of Brn3b mRNA relative to those expressing the scramble control following the inhibited expression of Fezf2 by the interference RNA (Fig. 5C). This data suggests that Brn3b depends on the transient expression of Fezf2 during retinal development.

To determine whether FEZF2 is a determinant of Brn3b expression in vivo, we also performed a gain-of-function study by in utero electroporation of Fezf2-pCAGEN into the developing retina. Expression of Fezf2 under a CAG promoter resulted in overall inhibition of RGC-related genes, including Math5, Isl1, and Brn3b (Fig. 6). A strong ectopic expression of this transcription factor may dramatically alter the phenotype of host cells. It was found that striatal progenitors change their fate from medium spiny neurons to cortex-like projection neurons after ectopic expression of Fezf2 (29). Intrinsic transient expression, but not a high and sustained expression of Fezf2, is essential for RGC development in the retina.

FEZF2 Binds to and Activates a 5′ Regulatory Region of Brn3b—Because both knockout and knockdown of Fezf2 decreased Brn3b mRNA in retinas (Figs. 3C and 4C), Fezf2 may regulate Brn3b at the transcriptional level. FEZF2 acted as a histone deacetylase-associated repressor down-regulating multiple oncogenes through direct binding to their promoters (30). In zebrafish, FEZF2 binds to a core consensus-binding site (CAAGCAACC) by which the transcription factor drives a forebrain enhancer activity of reporter genes both in vitro and in vivo (31). Four contiguous FEZF2 binding motifs are located at −274, −195, +21, and +211 of the transcriptional starting site of the Brn3b locus (Fig. 7A). We cloned this tentative regulatory region containing the Fezf2-binding motifs into pGL4.18, a luciferase reporter vector, for measuring promoter activity. The pGL4.18-Brn3b promoter was stably expressed in neuroblasto ma N2a cells, which were then transiently transfected with Fezf2-pCAGEN and pCAGEN or pLVTH-Fezf2-siRNA-1 and pLVTH-Fezf2-scRNA-1. The relative luciferase activity of Fezf2-transfected cells was 2.1-fold higher than that of the control. Conversely, the activity of the Fezf2-siRNA-transfected group was decreased by 51.6% compared with those of the scrambled control (Fig. 7B). The results indicate that this regulatory sequence near the 5′ UTR of Brn3b locus.

FIGURE 7. FEZF2 binds to the 5′ regulatory sequence in Brn3b. A, schematic of the genomic structure of Brn3b. A tentative promoter including the 5′ UTR of Brn3b contains predicted FEZF2 binding motifs (yellow bars). B, relative luciferase activities measured in the lysates of N2a cells. N2a cells stably expressing the pGL4.18-Brn3b promoter were assayed for luciferase activity after transient expression of the indicated constructs for 2 days. Fezf2-pCAGEN increased, whereas Fezf2-siRNA decreased, luciferase activity. C and D, ChIP with an anti-V5 antibody precipitated FEZF2-bound DNA from N2a cells expressing V5-tagged FEZF2 but not those expressing V5-GFP. The DNA was analyzed by gel electrophoresis (C) of the PCR products for the presence of the tentative promoter and measured by q-PCR (D) for enrichment of the binding sequence. The input of ChIP and the pulldown without V5 antibody were taken as positive and negative controls of the PCR, respectively. The control sequence for the q-PCR is D is a region in the 3′ UTR of the Brn3b locus.

Discussion

Retinal neurons start to generate from neuroepithelial cells in the optic cup during the middle of gestation. Newly generated neurons exit the cell cycle and migrate toward the inner (vitreal) side of the optic cup. By E13.5, the retina begins to transform from a uniform sheet of neuroepithelial cells into two
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layers of prospective GCLs and NBLs. This study examined the expression and function of Fezf2, a key transcription factor regulating cortical projection neurons, in the developing retina. We found that a peak of transient expression of Fezf2 mRNA occurs at E16.5 in the NBL, where the retinal progenitors are located (Fig. 1). Interestingly, Fezf2 protein was found in the GCL and colocalized with the RGC marker BRN3B (Fig. 2). The transient expression of Fezf2 in migrating retinal cells may allow its protein to accumulate in post-migratory neurons in the GCL. The pattern of Fezf2 expression is spatially analog to that of Math5. A knockin lacZ at the Math5 locus appeared in the retina, including the GCL (2), despite Math5 mRNA being expressed in the NBL at E15.5 (11). The expression of Fezf2 at E16.5 supports it as a later regulator of expressed in the NBL at E15.5 (11). The expression of BRN3B, despite and survival of RGCs (5, 6).

(Figs. 3 and 4), as expected for its function in the maintenance of BRN3B. The reduction of BRN3B is the cause, not the result, of the decrease in BRN3B (Fig. 5). This observation indicates that Fezf2 may not regulate peak formation (E11.5-E14.5) but help the survival of RGCs around E17.5, when maximal apoptosis occurs (5).

The roles of Fezf2 in the developing retina were examined by the study of loss of function. We found that Fezf2 knockout retinas had reduced BRN3B+ RGCs (Figs. 3 and 4). To determine whether this reduction is an effect of inhibition of Brn3b expression or developmental changes leading to the loss of BRN3B+ RGCs, we knocked down Fezf2 in a small population of retinal cells with RNA interference by in utero electroporation (18). The results supported the hypothesis that the knock-down of Fezf2 repressed BRN3B cell-autonomously. The inhibition of BRN3B was not accompanied by any loss of ISLET1 and BRN3A, two RGC markers that largely overlap with the expression of BRN3B (Fig. 5). This observation indicates that reduction of BRN3B is the cause, not the result, of the decrease in BRN3B+ RGCs in the knockout retina. The BRN3B deficit increased the apoptosis of RGCs and reduced optic nerve fibers (Figs. 3 and 4), as expected for its function in the maintenance and survival of RGCs (5, 6).

Our data revealed that Fezf2 was essential for the proper expression of BRN3B, whereas it had no effects on BRN3A (Fig. 5, D and E), a closely related protein in the family of class IV POU domain transcription factors, indicating a specific action of Fezf2 on Brn3b. A recent study found that the high-mobility group domain-containing transcription factors SOX4/SOX11 express in the developing mouse retina and function redundantly to regulate the generation and survival of RGCs (32). SOX4 and SOX11 transactivate Fezf2 by competing with the specific repressor SOX5 in the cerebral cortex (33). It is not clear whether SOX4 and SOX11 participate in the transient activation of Fezf2 in the retina. However, our results are consistent with the observation that targeted disruption of SOX4 or SOX11, positive regulators of Fezf2 (33), caused a reduction of RGCs in retinas (32).

The mechanisms responsible for FEZF2 regulation of Brn3b were explored in this study. A 5′ regulatory region of Brn3b containing a putative FEZF2 binding motif was enriched in the DNA-protein complex in N2a cells expressing V5-FEZF2 but not in those expressing V5-FEF2 (Fig. 7). This observation suggests that FEZF2 may bind to a tentative promoter of Brn3b. An in vitro assay found that FEZF2 activated a reporter construct carrying the 5′ regulatory part of Brn3b. The result indicates that FEZF2 enhances the transcription of Brn3b by binding to the genomic locus of Brn3b. Notably, the Wilms tumor gene Wt1, which encodes a zinc finger transcription factor, also activates the transcription from the 5′ regulatory sequence of Brn3b (34). The 5′ sequence, including the 5′ UTR, may contain positive control elements driving the transcription of Brn3b.

Mouse embryos with targeted disruption of Wt1 exhibit thinner retinas and enhanced apoptotic cell death of RGCs (34). This is in parallel to our observation that Fezf2-null retinas had increased apoptosis and reduced optic nerves (Figs. 3 and 4). Future studies are needed to determine whether Fezf2 knock-out leads to a visual deficiency in mice.

Although transient expression of Fezf2 is required for Brn3b, sustained expression completely blocked BRN3B (Fig. 6). The underlying mechanisms responsible for the inhibition of Brn3b are not clear. It remains to be excluded that the genomic locus of Brn3b may harbor unknown regions mediating inhibition by FEZF2. On the other hand, sustained expression of Fezf2 blocked MATH5 in addition to BRN3B and ISLET1 (Fig. 6). We speculate that Fezf2 could function as a repressor of Math5 and, thereafter, inhibit its downstream targets. It is known that targeted deletion of Math5 abolishes the retinal expression of Brn3b (2) despite Fezf2 expressing transiently in the developing retina. Therefore, if Math5 were depressed by a sustained Fezf2, then Brn3b would be blocked. With the inhibition of Math5, the phenotype of retinal cells may be altered by ectopic expression of Fezf2, a fate-determining gene of dorsal telencephalic progenitors and cortical projection neurons (17, 29).

In conclusion, we determined the expression and function of Fezf2 in the developing retina. Transient expression of Fezf2 is essential for the development and survival of BRN3B+ RGCs by regulating the transcription of Brn3b. This study provides novel insights into the molecular mechanisms underlying RGCs and retinal development.

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