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Ebf1 Deficiency Causes Increase of Müller Cells in the Retina and Abnormal Topographic Projection at the Optic Chiasm

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Abstract

The Ebf transcription factors play important roles in the developmental processes of many tissues. We have shown previously that four members of the Ebf family are expressed during mouse retinal development and are both necessary and sufficient to specify multiple retinal cell fates. Here we describe the changes in cell differentiation and retinal ganglion cell (RGC) projection in *Ebf1* knockout mice. Analysis of marker expression in *Ebf1* null mutant retinas reveals that loss of *Ebf1* function causes a significant increase of Müller cells. Moreover, there is an obvious decrease of ipsilateral and retinoretinal projections of RGC axons at the optic chiasm, whereas the contralateral projection significantly increases in the mutant mice. These data together suggests that Ebf1 is required for suppressing the Müller cell fate during retinogenesis and important for the correct topographic projection of RGC axons at the optic chiasm.

Keywords

Ebf1; Early B-cell Factor; Knockout; Retinal Development; Retinal Ganglion Cell; Axon Projection; Optic Chiasm

Introduction

The mouse retina is composed of six classes of neuronal cells, including the ganglion, amacrine, horizontal, bipolar, rod and cone cells, and one class of glia cells, the Müller cells. The seven classes of cells can be further divided into more than 50 subgroups with distinct morphologies and functions [1,2]. All retinal cell types arise from the same group of multipotent progenitors during retinogenesis. The process of retinal development is under the tight and delicate controls of both intrinsic and extrinsic factors [3,4]. The rod and cone photoreceptors reside in the outer nuclear layer and are responsible for collecting the light signal from the environment. The horizontal, bipolar and amacrine cells are interneurons located in the inner nuclear layer and are responsible for integrating and relaying the signal from the photoreceptors. The retinal ganglion cells (RGCs) integrate the signals from

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Competing Interests statement
The authors declare that they have no competing financial interests.

amacrine and bipolar cells, and then transmit them through their long exons, which bundle into the optic nerve and cross the optic chiasm before finally projecting into the brain.

Visual signals from each retina are transmitted to the thalamus and cerebral cortex on both sides of the brain. One of the crucial steps to establish the binocular and 3-dimensional vision is the proper RGC axon projection at the optic chiasm region. In the wild type mouse, there are about 3–5% RGC axons projecting ipsilaterally at the optic chiasm, while more than 95% RGC axons project contralaterally to the other side of the brain [5,6]. Interestingly, there is a small portion of RGC axons originated from the nasal part of the retina that project through the chiasm into the contralateral optic nerve, which is called the retinoretinal projection [7,8,9,10]. The retinoretinal projection normally disappears soon after birth, and its mechanism and developmental significance is still not clear. Whether RGC axons would extend to cross the midline at the optic chiasm, and at which direction they will further project into the brain, are under the dynamic control of interactions between a group of factors, such as EphB1/Ephrin B2 [11,12], NrCAM [13], Slit1/Slit2 [10], and transcription factors Zic2 [14,15], Foxd1 [16], etc.

The Ebf/Olf (Early B-Cell Factors) family of proteins are helix-loop-helix (HLH) transcription factors including four members, Ebf1 through Ebf4, that are important for neural development [7,17,18,19], adipogenesis [20,21], and lymphocyte development [22,23]. Previously we reported that all four members of the Ebf family are involved in retinal cell specification [24]. In the retinas, *Ebfs* are expressed in horizontal, ganglion, type 2 OFF-cone bipolar, and non-AII glycinergic amacrine cells [24]. However, the loss-of-function study with the knockout mice has not been done yet. Here we report the increase of Müller cell in the retina and abnormal RGC axon projection at the optic chiasm in the *Ebf1* null mouse, and reveal an essential role of *Ebf1* in regulating these developmental events.

Materials and Methods

Animals

All experiments with mice were performed in compliance with the IACUC protocols approved by the University of Medicine and Dentistry of New Jersey. The animals were housed and bred at the university facility. The C57BL/6J mice were obtained from the Jackson Laboratory. The *Ebf1* knockout mice were reported previously [22] and maintained by breeding with C57BL/6J mice.

In Vitro Retinal Explant Culture and Immunostaining

In vitro retinal explant culture, immunohistochemisty, and whole-mount immunostaining were performed as described previously [24,25,26]. The following primary antibodies were used: mouse anti-Brn3a (Millipore); goat anti-Bhlhb5 (Santa Cruz Biotechnology); rabbit anti-calbindin D-28k (Swant); sheep anti-Chx10 (Exalpha); rabbit anti-GABA (Sigma); mouse anti-glutamine synthetase (Millipore); goat anti-GLYT1 (Millipore); rabbit anti-NF150 (Millipore); rabbit anti- Pax6, (Millipore); rabbit anti-recoverin (Millipore); and rabbit anti-RxRg/RxRγ (Santa Cruz Biotechnology). Images were captured with either the Nikon Eclipse 80i Microscope or Leica TCS-SP2 Confocal Microscope.

DiI Labeling and Tracing

DiIC$_{18}$(3) (1,1′-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate) crystal was purchased from Invitrogen (Lot 454239). P0 mice were decapitated and fixed in fresh 4% PFA overnight. After the removal of lens and retina, a DiIC$_{18}$(3) crystal was placed onto the spot of the optic nerve The heads were kept at 37°C for 2 weeks in PBS with 0.05% sodium azide in a sealed cell culture plate. They were then dissected and the optic chiasm was exposed for imaging. All images were taken with the same parameters from the Nikon Eclipse 80i microscope.

Quantification

Retinas from the same litter were used for quantification analysis. Confocal images from matched regions of wild type and mutant retinas were captured with the same scanning thickness. At least 6 regions from each retina and 3 retinas from each genotype were captured. Marker-positive cells were scored from the images. All data were tested for significance using two sample Student's t-test with unequal variances.

The Dil labeling images were quantified using the NIH ImageJ software. First, use the freehand tool to select the region (ipsilateral, contralateral, or retinoretinal), then choose plugins → analyze → measure RGB. Record the value of the area and the average intensity of the red signal in the area. The percentage of contralateral, ipsilateral and retinoretinal projections was calculated by the relative value of (area × intensity).

Results

Early *Ebf1* Mutant Retinas Show No Obvious Cell Fate Changes

Ebf1 heterozygotes were crossed to obtain the homozygous mutant mice, which usually die soon after birth. To investigate whether loss of *Ebf1* would affect cell fates in early stage retinas, E13.5 and P0 retinas were examined. At E13.5, the size and morphology between wild type and mutant retinas are indistinguishable, and ganglion cells are unchanged in the mutants (data not shown). At P0, retinas were examined for any defects by immunostaining with antibodies against several retina cell markers, which include Pax6 for RGCs in the ganglion cell layer, amacrine and horizontal cells in the neuroblastic layer(Fig. 1A, B), Chx10 for bipolar and progenitor cells (Fig. 1C, D), Brn3a for RGCs (Fig. 1E, F), calbindin for horizontal cells and starburst and other amacrine cells (Fig. 1G, H), recoverin and RxRg (also called RxRγ) for rod and cone photoreceptors, respectively, in the outer neuroblastic layer (Fig. 1I, J, K, L); NF150 for horizontal cells in the inner neuroblastic layer and RGC axon fibers (Fig. 1M, N, O, P). None of the markers exhibited any obvious difference between wild type and *Ebf1* mutant retinas, suggesting that the absence of *Ebf1* does not affect the fates and differentiation of early retinal cell types. The normal nerve fiber patterns revealed by NF150 labeling imply that there are no intraocular RGC axon projection defects in the *Ebf1*$^{-/-}$ retinas.

Müller Cells Increase in the *Ebf1*$^{-/-}$ Retina

Since Ebf1 homozygous mutants die perinatally, we are unable to assess in vivo the development of late-born cell types in the mutant retina as most of them are not generated. To circumvent this problem, *in vitro* retinal explant culture was employed to examine any changes in cell fates and differentiation. P0 *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ retinas were dissected and cultured on filters for 8 days. We examined markers for major cell types, for instances, Pax6 (Fig. 2A, B) for ganglion, horizontal and amacrine cells, GABA (Fig. 2E, F) for GABAergic amacrine cells, calbindin (Fig. 2G, H) for horizontal and some amacrine cells, and Chx10 (Fig. 2K, L) for bipolar cells. There was no significant difference in the number of cells immunoreactive for these markers between wild type and mutant retinas. We have shown previously that Ebf1 is expressed in the glycinergic amacrine cells and type 2 OFF-cone bipolar cells [24]. Using GLYT1 as a glycinergic amacrine cell marker and Bhlhb5 as a type 2 OFF-cone bipolar cell marker, we found that neither cell type was obviously affected in the mutant retina (Fig. 2C, D, I, J). However, the number of Müller cells labeled by glutamine synthetase increased by about 27% (Figs. 2M, N; 3), suggesting that Ebf1 normally suppresses the differentiation of Müller glial cells.

Ebf1 Inactivation Causes Aberrant RGC Axon Projection at the Optic Chiasm

At the optic chiasm region, the majority of RGC axons cross the midline and project contralaterally to the other side of the brain; the remaining RGC axons (about 3–5% in the mouse) project ipsilaterally to the same side. Since *Ebf1* and other *Ebfs* are involved in the axon guidance and projection in other tissues [27,28,29,30], [27,28,29,30], it's possible that they may play similar roles in regulating the RGC axon projections in the retina and optic chiasm. As aforementioned, the intraocular projection is normal at P0 (Fig1O, P), indicating *Ebf1* deficiency doesn't affect RGC axon pathfinding and projection in the mutant retina. We then checked if the RGC axon projection was disturbed at the optic chiasm region in the P0 *Ebf1*$^{-/-}$ mice. At the optic chiasm of mutant mice, compared to the wild type, there are about 40% fewer RGC axons projecting ipsilaterally, and the RGC axons projecting retinoretinally to the contralateral optic nerve also decrease by more than 50% (Fig. 4); however, there is a significant increase of contralateral projection (Fig. 4). Thus, loss of *Ebf1* function leads to abnormal topographic projection of RGC axons at the optic chiasm.

Discussion

Ebf1 Influences Cell Specification and Differentiation in the Mouse Retina

Ebfs have been reported to influence cell differentiation and cell fates in lymphocyte genesis [22,23,31], in adipogenesis [20,21,32] and in neuronal differentiation [7,17,29,33], including specification of multiple retinal cell types and subtypes in the retinal development [24]. In the adult mouse retinas, Ebfs are expressed strongly in the RGCs, glycinergic amacrine cells, type 2 OFF-cone bipolar cells, and weakly in the horizontal cells; but not in GABAergic amacrine cells, photoreceptors, or Müller cells. Overexpression of Ebf1, Ebf2, or Ebf3 is sufficient to induce the glycinergic amacrine, type 2 OFF-cone bipolar, and horizontal cells in the mouse retina. Such effect can be successfully suppressed by the overexpression of a dominant-negative form Ebf1EnR [24].

In our current work, however, we found no obvious changes in glycinergic amacrine, bipolar and horizontal cells in *Ebf1*$^{-/-}$ mutants, which is not surprising. First, Ebf1, Ebf2, Ebf3 and Ebf4 have a near identical expression pattern and act redundantly in the retina [24], and it is highly possible that other *Ebfs* could compensate for the complete loss of *Ebf1* in the *Ebf1* null retina. Such a redundant role is evidenced by the observations in the brain. While there are olfactory neuron projection defects in Ebf2 and Ebf3 double heterozygous mice, the Ebf2 or Ebf3 single heterozygous mouse shows no such defects [27], since partial loss of *Ebf2* can be compensated by Ebf3, and *vice versa.* Another evidence from the opposite angle is that, *Ebf1*$^{-/-}$ embryos show specific defects in the embryonic striatum, where *Ebf1* is the only *Ebf* gene expressed [34]. Therefore, the loss of Ebf1 function in homozygous mutant retinas is very likely to be partly compensated by other Ebf factors in the neuronal differentiation process. Second, due to the limitation of the *in vitro* explant culture, there are a lot of cell deaths that would easily cover the minor differences between wild type and mutants. Using conditional knockout mouse of *Ebf1* should overcome the limitation. To avert the redundant effect and fully understand the role of Ebfs in retinal cell development, mouse with compound knockouts of two or more *Ebfs* should be examined for any possible specification defects.

In the cultured *Ebf1*$^{-/-}$ retinal explant, there is a 27% increase of Müller cells compared to the wild type, indicating that Ebf1 normally inhibits the Müller cell fate during retinogenesis. This is consistent with our previous gain-of-function analysis showing that misexpressed Ebf1 has a potent activity to suppress Müller cell differentiation [24]. The increase of Müller cells in the mutants should be a non-autonomous effect, since none of the Ebf factors is expressed in the Müller cells [24]. In addition, the role of Ebf1 to bias a neuronal *versus* a glial cell fate is also shared by many other bHLH factors, for exam ples, Mash1[25], NeuroD [35,36], Math3[35,36], Math5[37], Ngn1 & Ngn2 [38], etc.

Ebf1 Regulates RGC Axon Guidance and Projection

In various neural tissues, *Ebfs* have been shown to play an important role in controlling axon guidance and pathfinding. In the C. Elegans, mutants of the *Ebf* homolog *unc-3* have motor neuron axon pathfinding deficiency and display moving abnormality [30]. In the mouse olfactory bulb, knockout of *Ebf2* or *Ebf3* causes defects in olfactory axon projection [27]. In the cerebellum, null mutation of *Ebf2* leads to Purkinje cell migration defect [33]. *Ebfs* are expressed in newborn RGCs and continue their expression throughout the adult stage, suggesting that *Ebfs* may be involved in RGC axon guidance and projection, and other RGC routine functions. Our DiI tracing experiment demonstrated that Ebf1 does regulate the RGC axon projection.

Ebf1 null retina, the RGC axon fibers form normally, as seen by NF150 staining of P0 retinas (Fig1P). There are no obvious defects in the optic fissure or optic disk, and optic nerves exit the eyes normally. However, abnormal RGC axon projections do occur at the optic chiasm in *Ebf1* null mice. Compared to the wild type, *Ebf1* mutants exhibit more contralateral projection of RGC axons but less ipsilateral projection. The retinoretinal projection is also greatly reduced in the *Ebf1* mutant, a phenotype contrary to *Slit1/Slit2* double knockout mice[10], and *EXT1* null mice [39], suggesting Ebf1 has an opposing role against Slit1/Slit2 and EXT1 in regulating RGC axon projection. On the other hand, transcription factors Zic2 [14,15], Foxd1 [16]and Foxg1 [40,41] are the crucial proteins in regulating the ipsilateral and contralateral projections. Microarray analysis should give clues how these factors are affected in the *Ebf1* mutants.

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HILIGHTS

- Müller cell increase
- aberrant ipsilateral projection
- aberrant retinoretinal crossing

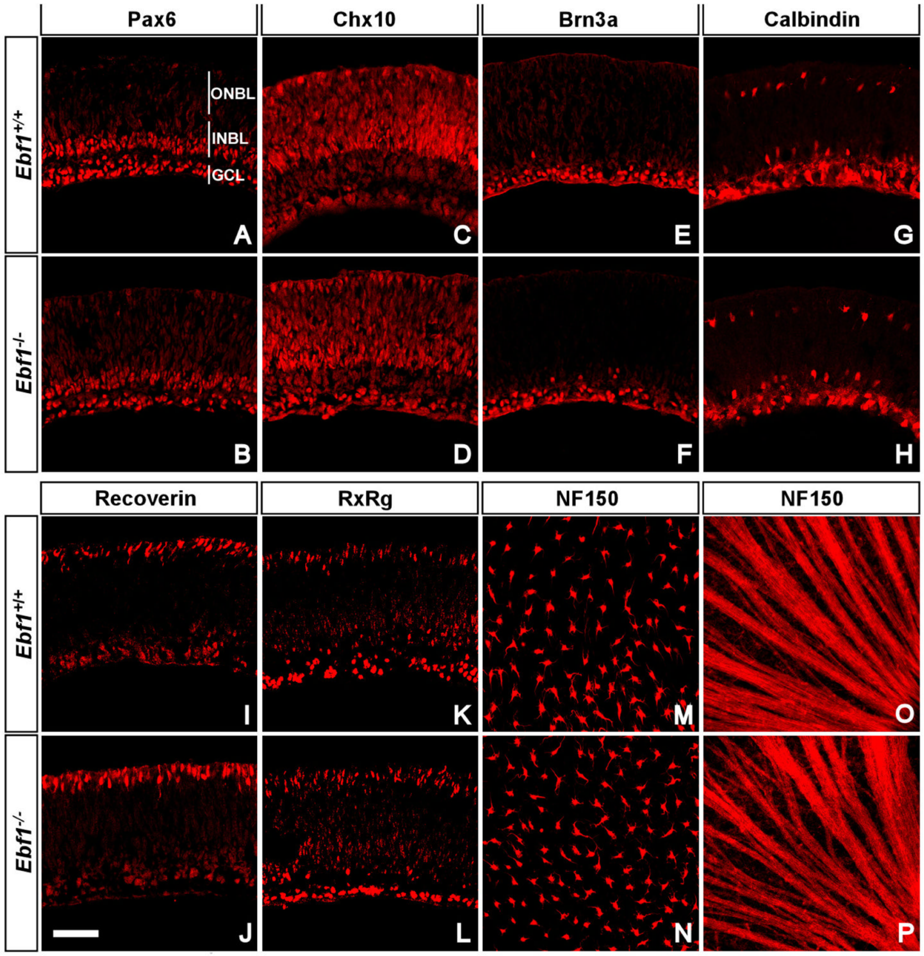

Fig. 1. Expression of cell markers in P0 *Ebf1*$^{+/+}$ and *Ebf1*$^{-/-}$ retinas

(A–L) P0 retinal sections from wild type and mutant retinas were immunolabeled with the indicated antibodies. **(A, B)** Anti- Pax6 stains RGCs, amacrine and horizontal cells. **(C, D)** Anti-Chx10 stains progenitor and bipolar cells. **(E, F)** Anti-Brn3a stains RGCs. **(G, H)** Anti-calbindin stains horizontal and amacrine cells. **(I, J)** Anti-recoverin stains photoreceptors. **(K, L)** Anti-Rxrg stains cone photoreceptors and RGCs. **(M–P)** NF150 whole-mount staining shows horizontal cells and RGC axon fibers at different layers. There are no significant changes in these markers between wild type and mutant retinas. **GCL,** ganglion cell layer; **INBL,** inner neuroblastic layer; **ONBL,** outer neuroblastic layer. Scale bar: **J** (for **A–P),** 50μm.

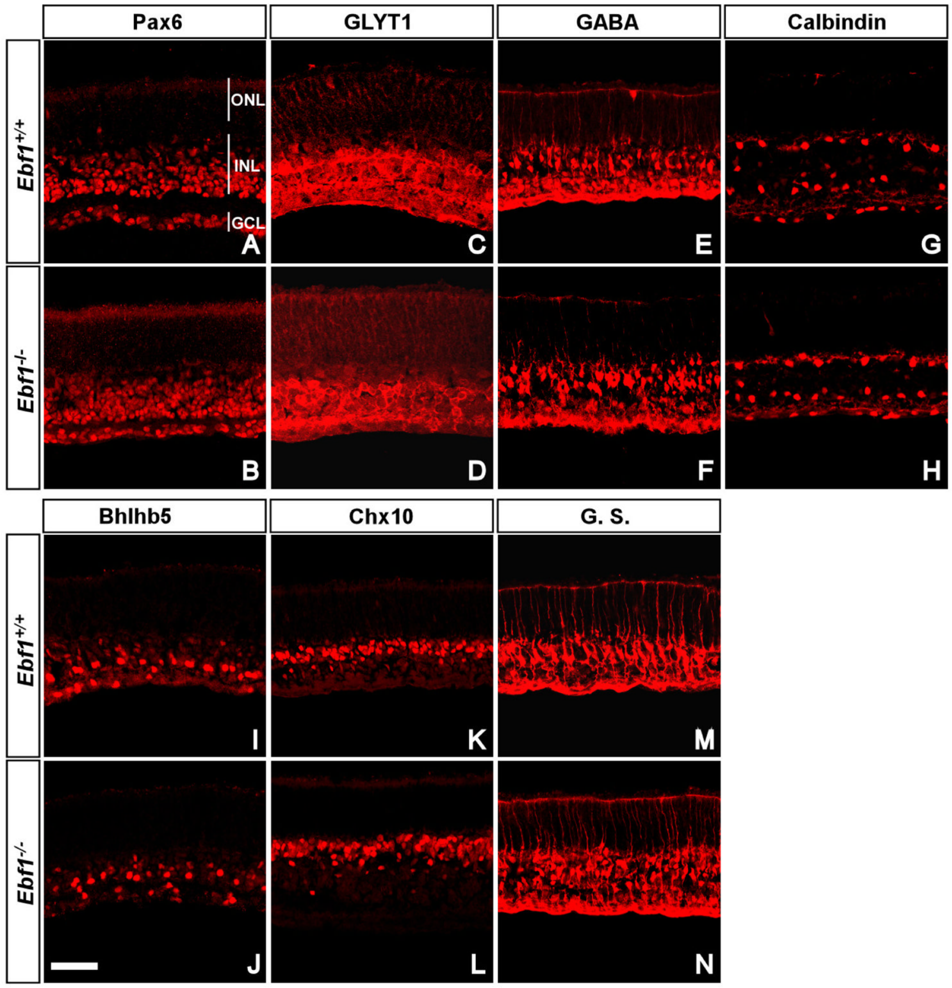

Fig. 2. Expression of cell markers in cultured P0 *Ebf*$^{+/+}$and *Ebf1*$^{-/-}$ retinal explants

(A–N) P0 Ebf$^{+/+}$and *Ebf1*$^{-/-}$ retinal explants were cultured in vitro for 8 days and their sections were then immunolabeled with the indicated antibodies. **(A, B)** Anti-Pax6 stains amacrine, horizontal cells and RGCs. **(C, D)** Anti-GLYT1 stains glycinergic amacrine cells. **(E, F)** Anti-GABA stains GABAergic amacrine cells. **(G, H)** Anti-calbindin stains horizontal and some amacrine cells. **(I, J)** Anti-Bhlhb5 stains type 2 OFF-cone bipolar cells and GABAergic amacrine cells. **(K, L)** Anti-Chx10 stains bipolar cells. **(M, N)** Anti-glutamine synthetase (G.S.) stains Müller cells. There are no obvious changes in these markers except the increase of G.S.-immunoreactive Müller cells labeled by G.S. **GCL,** ganglion cell layer; **INL,** inner nuclear layer; **ONL,** outer nuclear layer. Scale bar: **J** (for **A–N),** 50μm.

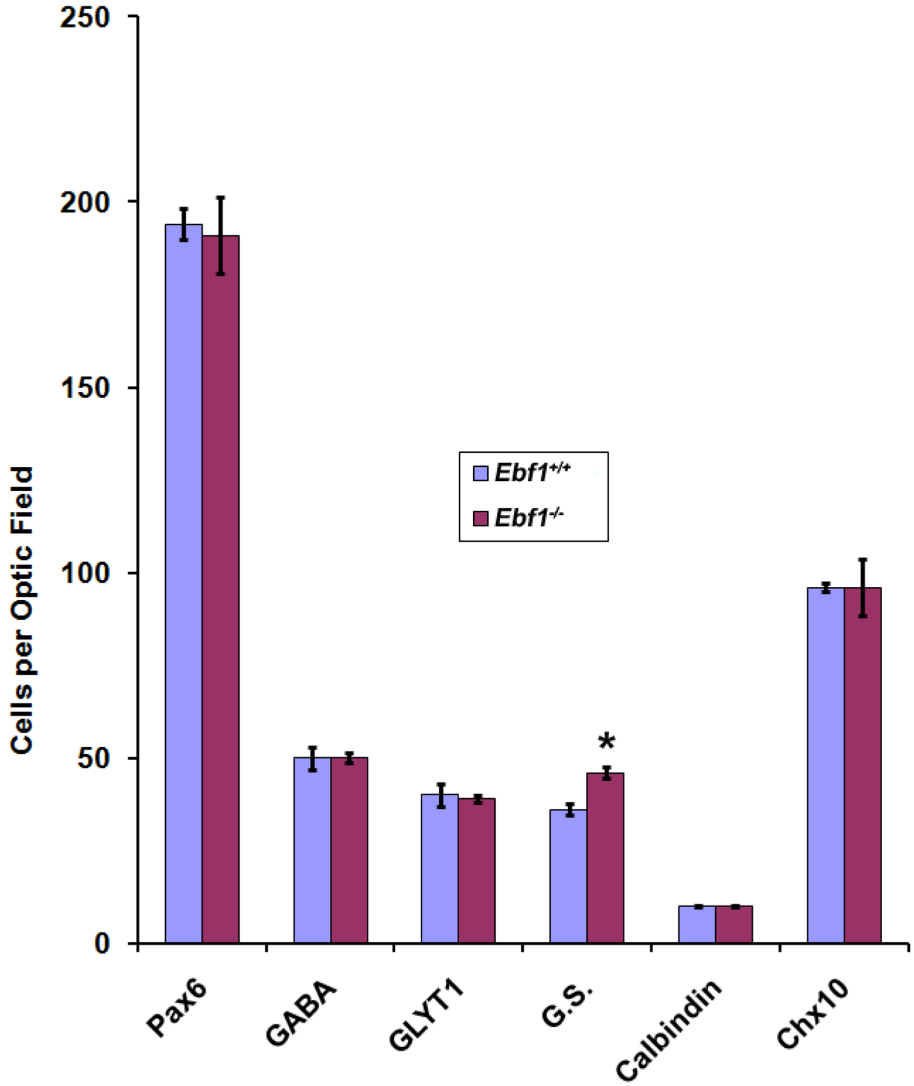

Fig. 3. Quantification of marker-positive cells in P0 *Ebf1*$^{+/+}$ *and Ebf1*$^{-/-}$ retinal explants
Marker- positive cells were scored and analyzed. There are no significant changes in these immunoreactive cell types between wild type and mutant retinas, except that G.S. Müller cells increase in the mutant. * p<0.05.

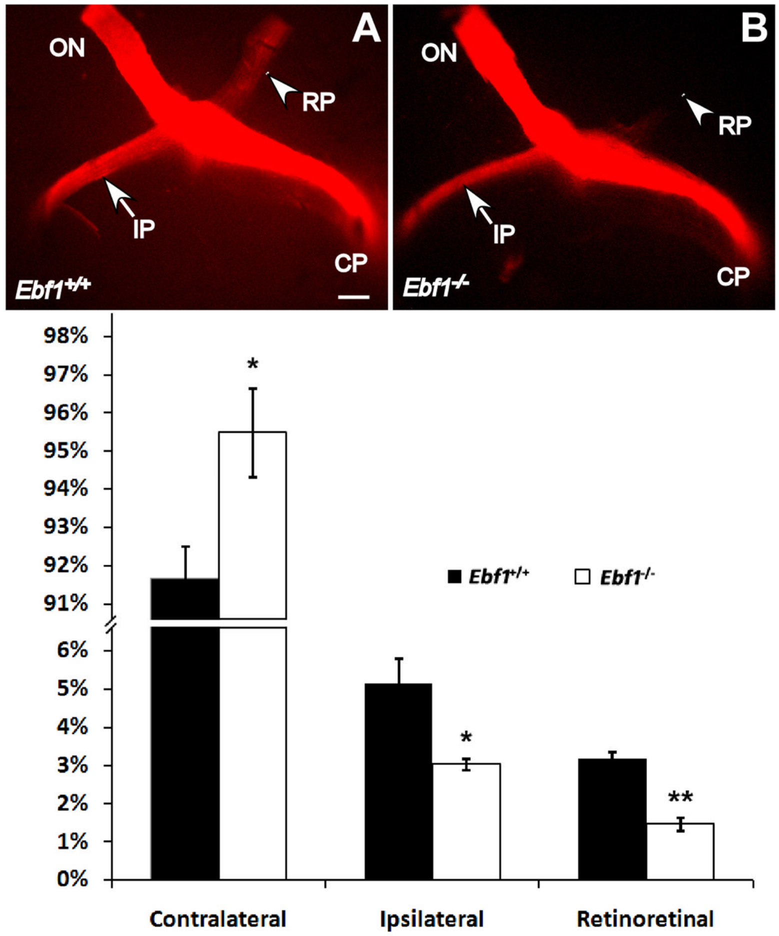

Fig. 4. RGC axon projection defects at the P0 *Ebf1*$^{-/-}$ optic chiasm

(A, B) In P0 *Ebf1*$^{-/-}$ m ice, there are fewer RGC axons projecting to the ipsilateral direction at the optic chiasm (pointed by the arrow), and even less axons projecting retinoretinally to the contralateral optic nerve (pointed by the arrowhead). **(C)** Percentage of axon projections. *P<0.05, **p<0.01. **CP,** contralateral projection; **IP,** ipsilateral projection; **ON,** optic nerve; **RP,** retinoretinal projection. Scale bar: **A** (for **A–B),** 500μm.