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Transcription factors SOX4 and SOX11 function redundantly to regulate the development of mouse retinal ganglion cells

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***Running title**: *Sox4* and *Sox11* in RGC development

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Background: Roles of *SoxC* genes in the development of retinal ganglion cells (RGCs) are unknown at present.
Result: Targeted deletion of *Sox4* and *Sox11* in retina results in a complete loss of RGCs.
Conclusion: *Sox4* and *Sox11* function redundantly to regulate RGC development.
Significance: These findings highlight the essential role of *SoxC* genes in retinal development.

SUMMARY

SOX family proteins belong to the high-mobility-group (HMG) domain-containing transcription factors, and function as key players to regulate embryonic development and cell fate determination. The highly related group C *Sox* genes *Sox4* and *Sox11* are widely expressed in the development of mouse retina and share a similar expression pattern with each other in this process. Here, to investigate the roles of *Sox4* and *Sox11* in the retinal development, *Sox4, Sox11* and *Sox4/Sox11* conditional knockout (CKO) mice with deletion of *Sox4, Sox11* and *Sox4/Sox11* in retinas were generated. Our studies demonstrated that targeted disruption of *Sox4* or *Sox11* in retinas caused a moderate reduction of generation of RGCs. However, a complete loss of RGCs was observed in *Sox4/Sox11-null* retinas, suggesting the two genes play similar roles in the development of RGCs. Our further analysis confirms that *Sox4* and *Sox11* function redundantly to regulate the generation of RGCs at early embryonic stages as well as the survival of RGCs at late embryonic stages. In addition, we demonstrated that loss of *Math5* impairs the expression of *Sox4* and *Sox11* in the ganglion cell layer while deletion of *Brn3b* has no effect on the expression of *Sox4* and *Sox11*. Taken together, these findings elucidate *SoxC* genes as essential contributors to maintain the survival of RGCs, and imply their intermediate position between *Math5* and *Brn3b* in the genetic hierarchy of RGC development.

The well-arranged laminar structure and easy accessibility of the mammalian retina make it an attractive model for studying the development of heterogeneous types of cells in a complex tissue. Mature mammalian retinas consist of six major types of neuronal cells and one type of glial cells, which are organized into three distinct cellular layers: photoreceptors (rods and cones) constitute the outer nuclear layer (ONL); horizontal, bipolar, amacrine, and Müller glial cells constitute the inner nuclear layer (INL) while ganglion and displaced amacrine cells constitute the ganglion cell layer (GCL). Among all seven major retinal cell types, retinal ganglion cells (RGCs), the only projection neurons in the retina, collect the signals and transmit them to the target locations within the brain (1,2). RGCs are the first-born neurons from multipotent retinal progenitor cells (RPCs) during retinogenesis. Formation of RGCs is a stepwise process involving extensive and precise molecular factors. MATH5 (ATOH7-Mouse Genome

Informatics) and BRN3B (POU4F2-Mouse Genome Informatics) are two of the most important transcription factors (TFs) involved in regulatory pathway of RGCs (3). MATH5, a basic helix-loop-helix (bHLH) TF, is expressed in post-mitotic RPCs and is required for maintaining the competency of RPCs to become RGCs (4,5). Loss of *Math5* in mice results in nearly complete absence of RGCs. As a POU-homeodomain (POU-HD) factor, BRN3B is one of TFs regulated by *Math5* (4,5). Different from the role of *Math5* in early stages of RGC specification, *Brn3b* functions in terminal differentiation and survival of RGCs by regulating axon outgrowth and apoptosis (6-9). Although it is clear that BRN3B functions downstream of MATH5, it remains unknown what other factors function between MATH5 and BRN3B to regulate RGC differentiation.

The Sry-related high mobility group (HMG) box (SOX) family of transcription factors is characterized by the highly conserved HMG motif and have been reported as critical regulators to control cell fate and differentiation in multiple processes during development (10,11). In mammals, the Sox family consists of more than 20 members, classified into eight subgroups A-H according to the sequence similarity among the HMG domains (12). Three *Sox* genes *Sox4*, *Sox11*, and *Sox12* belong to the mammalian *SoxC* group. *Sox12* knockout mice are viable and do not exhibit obvious abnormal phenotypes, but the deficiency of either *Sox4* or *Sox11* is lethal to mice (13-15). The broad expression of *SoxC* genes has been reported in neural progenitor cells and mesenchymal cells in variant tissues during development (13,14), and they have been found to regulate cell differentiation, proliferation and survival in multiple organ lineages (16-22). Recent studies on *Sox4* and *Sox11* imply their roles in retinal cell differentiation (23). However, the regulation of RGC development by the two genes has not been determined. Here we demonstrated that the expression of *Sox4* and *Sox11* was highly overlapping in the developing retina during embryonic stages. Targeted deletion of either *Sox4* or *Sox11* in the retina led to a moderate reduction of RGCs, whereas loss of *Sox4* and *Sox11* resulted in the absence of RGCs. Further analysis on the development of RGCs using three different conditional knockout (CKO) mice revealed that *Sox4* and *Sox11* function redundantly to govern the generation and survival of RGCs at early and late embryonic stages, respectively. Furthermore, a *Sox4/11-null* mutation did not affect the expression of *Math5* in retina, but removal of *Math5* abolished the expression of *Sox4* and *Sox11* in the GCL. In addition, we found that the deletion of *Brn3b* did not alter the expression of *Sox4* and *Sox11* in retina, and overexpression of *Sox4* or *Sox11* stimulated BRN3B expression *in vitro*. These studies strongly suggest that *Sox4* and *Sox11* function downstream of *Math5* while upstream of *Brn3b* to regulate the development of RGCs.

EXPERIMENTAL PROCEDURES

Animals - *Six3-cre* and $Sox4^{loxP/loxP}$ mice were described previously (24,25). To generate $Sox11^{loxP}$ allele, genomic sequences from mouse 129S6 BAC library was isolated using *Sox11*-coding sequences as a probe. The $Sox11^{loxP}$ targeting construct was created by inserting the *Sox11* 6.45 kb 5' flanking arm containing the open reading frame (ORF) and 3.0 kb 3' flanking arm into the 5' and 3' multiple cloning sites of the pKII-2FRT vector, respectively. In this construct, one loxP site was introduced before the ORF of *Sox11*, and the other loxP site was followed by an FRT-flanked Neo-cassette. Thus, the exon of *Sox11* could be removed by Cre recombinase. The targeting vector was linearized with *Not*I and electroporated into 129S6 mouse embryonic stem (ES) cells. Targeted ES cell clones were obtained by G418 positive selection and the negative selection conferred by diphtheria toxin A (DTA) gene. The selected ES cell clones were screened by Southern blotting with a 0.8 kb 5' probe which recognizes a 9.3 kb fragment of the wild-type allele and a 7.3 kb fragment of the targeted allele in genomic DNA digested with *Hind*III. Then the targeted ES cell lines were injected into C57BL/6J blastocysts to generate chimeras. Male chimeric mice were crossed with C57BL/6J female mice to generate F1 heterozygous mice whose genotypes were identified by Southern blotting. The primers for genotyping PCR were designed as follows: 5'-CGTGATTGCAAAGGCAGAGG and 5'-GTACTGAGGTCTAGGCTGTAAGG to detect a 500 bp product of wild-type *Sox11* allele and a 550 bp product of $Sox11^{loxP}$ allele; 5'-GAAGGAGGCGGAGAGTAGACGG and 5'-CATAGCTCAACACAAATGCCAACGC to detect a 407 bp product of wild-type *Sox4* allele and a 516 bp product of $Sox4^{loxP}$ allele; and 5'-GTGGAATCGCTGAATCTTGAC and 5'-GCCCAAATGTTGCTGGATAGT to detect *Six3-cre* allele. Embryos were defined as embryonic

day (E) 0.5 at noon on the day when vaginal plugs were detected. All procedures regarding animal work in this research were approved by University Committee of Animal Resources (UCAR) at the University of Rochester.

Hematoxylin and Eosin (H&E) staining, immunochemistry and in situ hybridization - For H&E staining, isolated eyes were fixed by immersion in 2.5% gluteraldehyde and 2% paraformaldehyde at 4°C for 24 hours. Then eyes were dehydrated and embedded in technovit, 2.5 μm sections were cut, and sections cut through the optic disc were stained with hematoxylin and eosin. For immunochemistry and *in situ* hybridization, staged mouse embryos and enucleated eyes of postnatal mice were dissected followed by immediate fixation in 4% paraformaldehyde in PBS for 1-2 hours at 4 °C. After fixation, tissue samples were cryopreserved with 25% sucrose, embedded and frozen in OCT medium. Horizontal cryosections were prepared at a thickness of 20 μm for *in situ* hybridization and 16 μm for immunochemistry experiments. BrdU labeling, immunostaining, and *in situ* hybridization were carried out as previously described (7). The following antibodies and working dilutions used in this study were: mouse anti-BRN3A (1:200; Millipore Bioscience Research Reagents), goat anti-BRN3B (1:200; Santa Cruz), mouse anti-SMI32 (1:1000; Sternberger Monoclonals), mouse anti-PAX6 [1:200; Developmental Studies Hybridoma Bank (DSHB)], sheep anti-CHX10 (1:200; Exalpha), mouse anti-SOX2 (1:200; Santa Cruz), mouse anti-CALBINDIN (1:2000; Sigma), rabbit anti-OPSIN (1:200; Sigma), mouse anti-RHODOPSIN (1:250; Cosmo Bio), mouse anti-BrdU (1:50; DSHB), rabbit anti-activated CASPASE-3 (1:500; R&D Systems), chicken anti-GFP (1:500; Abcam). Alexa-conjugated secondary antibodies (Invitrogen) were used at a dilution of 1:1000. *Sox4* and *Sox11* probes were generous gifts from Dr. Paul J. Scotting (26). Confocal images were captured with a Zeiss LSM 510 META confocal microscopy. Other pictures were taken with a Nikon Eclipse TE2000-U inverted microscope. Acquired pictures were quantitatively analyzed by manually counting specific marker-stained cells.

Plasmids, in vitro electroporation and retinal explant culture - The ORF of *Sox4* and *Sox11* were synthesized by Genewiz and cloned into the backbone of the pEGFP-N1 (Clontech) plasmid using *Sal*I and *Not*I restriction enzymes. pcDNA3 was used as a control plasmid. Retinas at E13.5 from C57BL/6J mice were dissected and transferred to a micro electroporation chamber (Harvard Apparatus #45-0105) containing a DNA solution at the final concentration of 1 μg/ μl in 1X PBS. The setting of electroporation was as follows: mode, LV; voltage, 30 V; pulse length, 50 msec; number of pulses, 5; interval, 950 msec; polarity, unipolar. After electroporations, retinas were cultured on Millipore Millicell-CM Low Height Culture Plant Inserts (0.4 μm pore size) in explant culture medium [45% DMEM, 45% HAM F-12 (Bio-Whittaker), 10% fetal bovine serum, 1X pennicilin/streptomycin/L-glutamine (Invitrogen), and 1X insulin (Sigma)] at 37°C in a humidified 5% incubator. Retinal explant samples were collected after 48 hours of culture.

RESULTS

Overlapping expression of Sox4 and Sox11 during the development of RGCs - To better elucidate the roles of *Sox4* and *Sox11* in RGC development, we compared their spatiotemporal expression patterns in embryonic retinas from E10.5 to E18.5. *In situ* hybridization revealed no detectable *Sox4* expression at E10.5 (Fig. 1A) and its expression first appeared in a small patch of cells in the central, dorsal optic cup at E11.5 (Fig. 1B). As retinal development progressed, *Sox4* expression rapidly expanded circumferentially towards peripheral retinal regions from E12.5 to E14.5 (Fig. 1C, D, I). *Sox4*-expressing cells was distributed in two layers with different levels: dispersive expression in the neuroblast layear (NBL) and constitutively higher level in the GCL. Besides the GCL, robust expression of *Sox4* was also found in the inner most parts of the NBL at E15.5 (Fig. 1J). From E16.5 to E18.5, a slightly intensive expression of *Sox4* was detected at the outer boundary of the NBL as well (Fig. 1K, L; data not shown).

In contrast to *Sox4*, modest expression of *Sox11* had been widely observed in the optic cup at E10.5 (Fig. 1E), which was consistent with the previous report (27). There was a mild increase in expression at E11.5, especially in the central region of the neural retina (Fig. 1F). At E12.5, *Sox11*-expresssing cells were distributed throughout the retina with highest expression level in the center (Fig. 1G). From E13.5 to E14.5, the expression of *Sox11* was identical to that of *Sox4,* with a higher level in the GCL and a lower level in the NBL (Fig. 1H, M). From E15.5 to E18.5, the

expression of *Sox11* was similar to that of *Sox4* (Fig. 1N, O, P; data not shown). Scattered expression of *Sox11* was detected in the NBL with moderate expression at the outer boundary and strong expression in the GCL. Together, *Sox4* and *Sox11* share a comparable expression pattern during retinal development, especially in the GCL. Their intensive expression starts from E11.5 in a central-to-peripheral wave and persists in the GCL during the generation of RGCs, indicating *Sox4* and *Sox11* may play redundant roles in the development of RGCs.

Targeted disruption of Sox4 and Sox11 in retina - Conventional *Sox11* knockout mice died immediately at birth due to the congenital cyanosis resulting from heart defects and hypoplasia of the lungs (28). To examine the function of *Sox11* in retinal development, we generated a *Sox11* CKO allele ($Sox11^{loxP}$) by flanking the entire open reading frame (ORF) with loxP sites (Fig. 2A). Therefore, the exon of *Sox11* could be removed by Cre recombinase. The genotypes of $Sox11^{loxP}$ mice were verified by Southern blotting and PCR (Fig. 2B, C). To remove *Sox11* in the retina, we used *Six3-cre* mice, which express Cre recombinase in the eye field and ventral forebrain starting at E9 to E9.5 (24,29). Homozygous $Sox11^{loxP/loxP}$ mice were bred with *Six3-cre* mice to yield retina-specific *Sox11* conditional knockout (CKO) ($Sox11^{loxP/loxP}$; *Six3-cre*) mice. *In situ* hybridization confirmed the efficient removal of *Sox11* in the retinas of $Sox11^{loxP/loxP}$; *Six3-cre* mice at E14.5 (Fig. 2D). In the following experiments, $Sox11^{loxP/loxP}$; *Six3-cre* mice were defined as *Sox11*-nulls. $Sox11^{loxP/+}$ and $Sox11^{loxP/loxP}$ mice were phenotypically indistinguishable and were used as controls hereafter. *Sox11*-nulls displayed a moderately reduced body size compared with controls at birth. Nevertheless, they reached body weight and size similar to those of the controls after postnatal day (P) 30 and were viable and fertile.

Given the fact that *Sox4* and *Sox11* share similar structures and expression patterns in the retinal development, *Sox4* could compensate for the loss of *Sox11*. Since conventional *Sox4* knockout mice die at around E14 from the severe malformation of the heart outflow tract (15), we used $Sox4^{loxP/loxP}$ mice (25), and generated the *Sox4-null* and *Sox4/11-null* mice using *Six3-cre* as mentioned above. *Sox4*-nulls were viable and fertile with no overt discernible physical deficiencies. While *Sox4/11-null* mice exhibited a smaller body size than the controls from birth to death before postnatal day (P) 14.

Retinal defects in Sox4/11-null mice - To examine the possible defects of RGCs in *Sox4*-, *Sox11*- and *Sox4/11-null* retinas, we analyzed the retinal sections at P30 from *Sox4-null* and *Sox11-null* mice, and at P14 from *Sox4/11-null* mice. H&E staining revealed that deletion of either *Sox4* or *Sox11* did not alter the retinal structure. The gross organization of *Sox4-null* or *Sox11-null* retinas resembled that of the control retinas, while the thickness of the GCL and the INL was reduced in either *Sox4-null* or *Sox11-null* retinas compared to those of the control retinas (Fig. 3A-A', F-F'). In adult mice retinas, BRN3A and BRN3B label about 70% RGCs separately in a partly overlapping pattern (30). Immunostaining with BRN3A showed fewer RGCs in either *Sox4*- or *Sox11-null* retinas in comparison with that of the control retinas in the absence of *Sox4* or *Sox11* (Fig. 3B-B', G-G'). Similar to BRN3A labeling, whole-mount immunostaining revealed a reduction of BRN3B+ RGCs in *Sox4* or *Sox11* nulls (Fig. 3C-C', H-H'). Quantification of BRN3B+ exhibited approximately 21% and 28% reduction of RGCs in *Sox4-nulls* and *Sox11-nulls*, respectively (Fig. 3P, Q). To confirm the loss of RGCs in mutants, SMI32, which predominantly marks large RGCs and their nerve fibers (31), was used as another parameter of RGC number. Immunostaining of whole mount retinas with SMI32 revealed that not only *Sox4*- or *Sox11-null* retinas had a obviously less number of axon bundles but also their axon bundles was less fasciculated (Fig. 3 D-D', I-I'). Consistently, optic nerves, which are composed of RGC axons, were thinner in *Sox4*- or *Sox11-null* retinas compared to that of controls (Fig. 3E-E', J-J'). Therefore, deletion of either *Sox4* or *Sox11* resulted in a moderate reduction of RGCs. However, removal of both *Sox4* and *Sox11* completely disrupted the overall structure of retinas. Compared with control retinas, the three nuclear layers were reduced to one thin layer, the entire inner plexiform layer and outer plexiform layer were abolished in *Sox4/11-null* retinas (Fig. 3K-K'). Furthermore, neither BRN3A+ nor BRN3B+ cells were observed in *Sox4* and *Sox11* compound null retinas (Fig. 3L-L', M-M', N-N'). The ventral views of mouse brains showed that optic nerves were absent in *Sox4/11-null* retinas as well (Fig. 3O-O'). Taken together, these data demonstrate that deletion of both *Sox4* and *Sox11* in retinas abolishes RGCs, implying the redundant function of *Sox4* and *Sox11* in RGC development.

That retinal inactivation of *Sox4* or *Sox11*

leads to a reduced thickness of GCL and INL suggests the potential absence or degeneration of other retinal type cells in addition to RGCs. To test this possibility, immunostaining using distinct cell type-specific markers were performed in retinal sections from mice at P30. Immunostaining with PAX6, a pan-amacrine cell marker, revealed about a 21% reduction of PAX6+ cells in the INL and a 43% reduction of PAX6+ cells in the GCL of *Sox4-null* retinas, respectively (Supplementary Fig. 1A-A', S). Immunostaining with CHX10 for pan-bipolar cells revealed a 26% reduction of bipolar cells in *Sox4-null* retinas (Supplementary Fig. 1B-B', S). In *Sox11* mutant retina, anti-PAX6 labeling showed that amacrine cells were decreased ~28% in the INL and ~37% in the GCL (Supplementary Fig. 1G-G', T), and anti-CHX10 labeling revealed ~33% reduction of bipolar cells (Supplementary Fig. 1H-H', T). Whereas, no significant differences in horizontal (CALBINDIN+), Müller glial (SOX2+ cells in the middle parts of INL), cone (OPSIN+) and rod (RHODOPSIN+) cells were detected between *Sox4-null* or *Sox11-null* and control retinas (Supplementary Fig. 1C-F, C'-F', I-L, I'-L', S, T). Although deletion of *Sox4* and *Sox11* results in one thin layer of retina, immunostaining with different cell type-specific markers revealed that all retinal cell types mentioned above were present but in much fewer numbers in *Sox4/11-null* retinas (Supplementary Fig. 1M-R, M'-R').

Requirement for Sox4 and Sox11 in the development of RGCs - In mice, RGCs are generated from E11.5 to E18.5 (32). BRN3B, whose expression initiates at E11.5, is one of the earliest RGC markers (33,34). The onset of robust expression of *Sox4* and *Sox11* in the retina coincides with the differentiation of RGCs in mice, implying their essential roles in RGC differentiation. Thus, we examined the expression of BRN3B in *Sox4-*, *Sox11-* and *Sox4/11-null* retinas at different embryonic stages. Immunostaining with BRN3B showed no substantial change in number and distribution of RGCs at E12.5 and E14.5 in *Sox4-null* retinas in comparison to that of control retinas (Fig. 4A-A', B-B', J), but at E16.5 the *Sox4* mutant retinas revealed a mild but significant reduction of BRN3B+ RGCs by ~20% (Fig. 4C-C', J). Different from *Sox4-null* retinas, *Sox11-null* retinas exhibited similar labeling pattern but reduced number of RGCs, particularly in the GCL, from E12.5 to E16.5 compared to that of control retinas (Fig. 4D-D', E-E', F-F'). Quantification analysis further confirmed that the declines were significant in *Sox11* mutants (Fig. 4K). Additionally, the number of BRN3B+ RGCs at P0 in *Sox4-* or *Sox11-null* mutants decreased by 27% or 28% respectively (Fig. 4J, K), which is comparable to the reduction in adult retinas (Fig. 3P, Q). However, the combined absence of *Sox4* and *Sox11* leads to a severe hypoplasia of the developing RGCs. At E12.5, only few BRN3B+ cells were detected in the mutant (Fig. 4G-G'). In contrast to control retinas, the GCL with BRN3B+ cells was much thinner at E14.5 and eventually disappeared at E16.5. At both E14.5 and E16.5, BRN3B+ cells mainly resided in the NBL (Fig. 4H-H', I-I'). By quantifying the number of BRN3B+ cells at E14.5 and E16.5, we found that the number of RGCs in double mutants remained unchanged, which was distinguished from the increasing trend in control retinas (Fig. 4L). In addition, we observed that deletion of *Sox11* led to a mild reduction in size of retinas, while deletion of both genes resulted in evident change in retinas at early embryonic stages, indicating that they possibly have impacts on the RPCs. Indeed, immunostaining with progenitor markers, CHX10 and SOX2, confirmed that the two genes affect the RPCs at early embryonic stages (data not shown).

It is possible that the gradual loss of BRN3B+ RGCs was caused by increased apoptosis in mutant retinas. Therefore, we examined the number of CASP3+ cells in control and mutant retinas. Previous research has shown that apoptosis is rare throughout embryogenesis (35,36). We found no significant change of the number of apoptotic cells at E12.5 in both control and mutant retinas (data not shown). However, starting from E14.5, expression of CASP3 was upregulated in all three mutants compared to controls (Fig. 5A-F, A'-F'). Quantification analysis revealed that *Sox4-* or *Sox11-null* mutants had an approximately 2-fold increase in the number of apoptotic cells, while ~ 6-fold and more than 10-fold increase were found in *Sox4/11-null* mutant at E14.5 and E16.5, respecitively (Fig. 5G, H, I). Moreover, compared to *Sox4-* or *Sox11-null* retina, more cells undergoing apoptosis were detected in the GCL of *Sox4/11-null* retinas at E14.5 (Fig. 5A', C', E'), the time consistent with the extensive loss of BRN3B+ RGCs.

Taken together, deletion of either *Sox4* or *Sox11* has no or a minor influence on the formation of most RGCs, suggesting that either of them is dispensable for the development of major RGCs. However, removal of both genes results in

a severe hypoplasia of RGCs at early stages and progressive loss of RGCs at late stages, indicating they play similar roles in different time windows during the development of RGCs: *Sox4* and *Sox11* are essentially required for the generation of primary RGCs at early stages and maintaining RGC survival during late stages.

Roles of Sox4 and Sox11 in the genetic regulatory network of RGCs - Previous studies have identified an essential *Math5→Brn3b* pathway in the development of RGCs (4,5). To determine the relationship between *SoxC* genes and *Math5* or *Brn3b* in the RGC genetic hierarchy, we examined the expression of *Sox4* and *Sox11* in *Math5-null* or *Brn3b-null* retinas, separately. Compared to control retina, both *Sox4* and *Sox11* expression was reduced in the NBL and was abolished in the GCL of *Math5-null* retina at E13.5 (Fig. 6A, B, C, D), indicating that *Math5* acts upstream of *Sox4* and *Sox11* during RGC development. In contrast, targeted deletion of *Brn3b had* no effect on the expression of *SoxC* group genes in retinas at E14.5 (Fig. 6E, F, G, H). Our findings suggest that *Sox4* and *Sox11* likely function between *Math5* and *Brn3b* to regulate RGC development. Next, we tested whether the deletion of *SoxC* could influence the expression of *Math5*. As shown by *in situ* hybridization, similar to controls, normal onset of *Math5* expression was detected in all three mutants including the *Sox4/11-null* mutant (Fig. 6I-K, I'-K'), further confirming that *Sox4* and *Sox11* function downstream of *Math5*.

To further test roles of *Sox4* and *Sox11* in the development of RGCs, gain-of-function experiments in embryonic retinal explant cultures were conducted. C57BL/6J mouse retinas at E13.5 were dissected and electroporated with vectors pCMV-*Sox4* or pCMV-*Sox11* and the pEGFP-N1 vector in a ration of 1:1. Retinal explants were harvested and examined after 2 days of culture. Overexpression of either *Sox4* or *Sox11* in retinas significantly increased the percentage of BRN3B+ cells by approximately 30% in comparison to pcDNA3 control vector electroporated retinas (Fig. 6N-N', Q-Q', R), indicating *SoxC* group genes enhance the generation of RGCs. We also observed robust expression of GFP, which served as the internal control, in both control and experimental retina sections (Fig. 6L-L', O-O'), indicating that a high electroporation efficiency was consistently achieved and the possibility that fluctuated electroporation efficiencies affected results was ruled out. In summary, our data indicates that *SoxC* group genes are essential intermediate factors between *Math5* and *Brn3b* to promote the development of RGCs.

DISCUSSION

In this report, we generated *Sox4*, *Sox11*, and *Sox4/11* CKO mice with specific deletions in retinas. Using the three mice models, our studies demonstrate that *Sox4* and *Sox11* are essential factors orchestrating the development of RGCs by promoting the differentiation and survival of RGCs. Previous studies on their potential functions in development have been mainly focused on their roles in maintaining the differentiation ability of progenitor cells and promoting cell proliferation. Overexpression of *Sox4* or *Sox11* in cultured retinal explants at E17 stimulates the differentiation of progenitor cells into cone cells at the cost of losing rod cells (23). Abrogation of *Sox4/11 in vivo* suppresses the differentiation of adult neuron stem cells and does not affect the apoptosis (20). In addition, knocking down of *Sox4* is reported to damage proliferation of osteoblasts *in vitro* (37). The proliferation of pancreatic islet cells is also impaired in *Sox4-null* pancreas cultured *in vitro* but *Sox4* deficiency has no impact on cell survival of insulin producing cells (38). However, it has been reported that *Sox4* and *Sox11* are upregulated in several types of tumors (39,40), indicating their roles in anti-apoptosis or pro-survival. In recent research, attention has been paid to their pro-survival roles in the development of mice. Ablation of both factors greatly reduced the survival of neural and mesenchymal progenitor cells during organogenesis (17), as well as of neuron progenitor cells during the spinal cord development (22). While *Sox11* was reported to play an indispensable role in the proliferation of tyrosine hydroxylase expressing cells at early stages, *Sox4* is crucial to their survival at late stages (21). Further lines of evidence demonstrate that *Sox11* also helps improve the survival of differentiated post-mitotic sensory neurons (19). Our data about the regulation of RGC development, for the first time, provides direct evidence indicating that *Sox4* and *Sox11* not only share functions in controlling differentiation and proliferation but also work in the same pathway of regulating cell survival of post-mitotic RGCs.

Targeted deletion of either *Sox4* or *Sox11* in RGCs results in a moderate inhibitory effect on RGC survival. However, loss of both genes results in dramatic increase in the number of CASP3+

RGCs during development in comparison to controls, demonstrating that they play redundant roles in promoting the survival of RGCs. Why do the two genes have redundant functions? The *SoxC* group comprises 3 genes, *Sox4*, *Sox11* and *Sox12*. They are structurally similar to one another with a highly conserved HMG box and a less conserved transcriptional activation domain (13). Interesting, very similar to the phenotype of *Sox11*$^{-/-}$ mice, *Sox4*$^{+/-}$*; Sox11*$^{+/-}$ mice died at birth from heart malformation, suggesting the dosage of the two genes are essential to heart development and they might function redundantly (17). During the development of the spinal cord, deletion of either *Sox4* or *Sox11* had no significant effect on the cell survival in the spinal cord, but loss of both reveals an up to a 70% decrease of cell number, strongly supporting the hypothesis that *Sox4* and *Sox11* regulate certain biological processes simultaneously (22). In comparison to *Sox4* and *Sox11*, although the expression pattern of *Sox12* in certain developing tissues including lung, gut and pancreas is similar, the biochemical property of weak binding activity to promoter DNA makes *Sox12* a less important factor in the regulation of development (13). And indeed, no phenotype of *Sox12* knockout mice was observed (14). In addition, our study shows that abolishment of both *Sox4* and *Sox11* by *Six3-cre* recombinase disrupts the formation of RGCs by 80% at E16.5 and leads to the complete loss of RGCs at later stages, suggesting *Sox12* either does not participate or plays a limited role in regulating the development of RGCs.

Moreover, in the present study, we delineated the function of *Sox4* and *Sox11* in the genetic pathway regulating the generation of RGCs. Previous research has established that *Math5* and *Brn3b* are two pivotal TFs in controlling development of RGCs (3). In the mouse retina, the expression of *Math5* first appears at E11 and its expression expands circumferentially to the peripheral retina from E11-16.5 (4,41). Its expression is limited to the RPCs and the nascent, migrating BRN3B+ RGCs in the NBL but is turned off in the post-migration RGCs in the GCL. MATH5 likely promotes the cell cycle exit of the RPCs as *Math5*-null mutation causes the failure of the progenitors to exit cell cycle (42). *Math5* determines RGC competence acquisition but does not specify the fate of RGCs (5). Moreover, *Brn3b* functions in the terminal differentiation and survival of RGCs (7). The factors responsible for turning RGC-competent progenitors into post-mitotic RGCs are yet to be identified. Based on the spatiotemporal expression pattern of *Sox4* and *Sox11* in the NBL as well as in the GCL during early retinogenesis, we hypothesized that *Sox4* and *Sox11* are the genes promoting RGC fate commitment. Expressions of *Sox4* and *Sox11* have been shown to be down-regulated by approximately 30% and 50% in *Math5*$^{-/-}$ retinas at E14.5 in comparison to control using microarray analysis (29), however, no further study was conducted to confirm the finding. Our analysis using *Math5-null* mutant provides direct evidence proving that loss of *Math5* greatly impairs the expression of both *SoxC* genes in the GCL. On the contrary, loss of *Sox4/11* does not alter the expression of *Math5* in retinas at E12.5. Therefore, *Sox4* and *Sox11* function downstream of *Math5* in RGC development. *Math5* starts to express in retinas at E11 and our findings show that in the control retinas at E11.5, the number of RPCs is 1.5-fold higher than that in *Sox4/11-null* retinas whereas interestingly early RGCs generated at E12.5 in the control is ~18-fold higher than those in *Sox4/11-null* retinas, strongly favoring that after *Math5* endows RPCs with RGC competence, *Sox4* and *Sox11* are necessary for specification or early differentiation of RGCs. The increased generation of RGCs observed in *Sox4* and *Sox11* overexpression further supports the role of *SoxC* genes in RGC differentiation. Whether MATH5 as a TF directly targets the promoter of *Sox4* and *Sox11* to regulate their expression is still unclear. A further experiment using ChIP assay would help answer this question. In addition, no apparent change of *Sox4* or *Sox11* expression is observed in the retina lacking *Brn3b* at E14.5, indicating that *Sox4* and *Sox11* unlikely acts downstream of *Brn3b* in RGC development. In contrast, loss of *Sox4* and *Sox11* alone or together causes a reduction or loss of BRN3B+ RGCs, arguing for a role of *Sox4* and *Sox11* upstream of *Brn3b* but downstream of *Math5* in the *Math5*→*Brn3b* regulatory pathway of RGC development (Fig. 6S). The moderate increase in the number of BRN3B+ cells conferred by *Sox4* or *Sox11* overexpression further supports the role of *Sox4* and *Sox11* upstream of *Brn3b*.

In *Sox4/11*-null retinas, BRN3B+ RGCs die from apoptosis beginning at E14.5, indicating that *Sox4/11* promotes RGC survival by either directly regulating apoptosis or mediating the regulation of apoptosis by *Brn3b*. Deletion of *Brn3b* starts to have a negative impact on RGC survival as late as E16.5 (33,43) while loss of *Sox4/11* starts to

suppress the survival at the stage of E14.5, suggesting that *Sox4/11* attains a pro-survival function independently of *Brn3b*. It is still plausible that *Sox4/11* may mediate the regulation of RGC survival by *Brn3b*.

Compared to the *Math5*-null or *Brn3b*/*Isl1*-null retinas with a loss of nearly all RGCs (4,9), the defect observed in *Sox4/11*-null retinas is more severe with a more profound reduction in other retinal cell types in addition to RGCs, suggesting a possible, direct involvement of *Sox4* and *Sox11* in the differentiation or survival or both of other retinal cell types. Consistent with this, the expression of *Sox4* and *Sox11* continues after the peak of RGC genesis in the GCL and NBL (Fig. 1) and persists until at least P11 in the developing INL (data not shown). Future experiments using specific Cre deleter mouse lines to remove *Sox4* and *Sox11* after RGC development will help define their role in the development of other retinal cell types.

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FIGURE LEGENDS

FIGURE 1. **Expression profiles of *Sox4* and *Sox11* during retinogenesis.** Wild type retinas at different embryonic stages (E10.5-E17.5) were collected for *in situ* hybridization using probes against *Sox4* (A-D, I-L) and *Sox11* (E-H, M-P). L: lens, R: retina, GCL: ganglion, NBL: neuroblast layer. Scale bar: 100 μm.

FIGURE 2. **Generation of a *Sox11* conditional null allele**. **(A)** Schematic representation of a portion of the wild type *Sox11* allele, the targeting construct and the targeted alleles. Grey filled box is the open reading frame (ORF) of *Sox11*. Black filled boxes are the sequences used to generate the homologous arms in the targeting vector. The $Sox11^{CKO}$ targeting vector is made by inserting the FRT-flanked neomycin gene and one loxP site before the ORF of *Sox11*. Cre recombinase mediated deletion of the loxP-flanked *Sox11* ORF results in a *Sox11* null mutation. Blue filled box is the 5' hybridization probe used for Southern blot analysis. Abbreviations: Neo, PGK-neomycin resistance gene; DTA, diphtheria toxin gene for negative selection of embryonic stem cells; loxP, Cre recombinase recognition sequence. **(B)** Southern blot analysis of targeted ES cells. A 5' probe was used in Southern blot of *Hind*III-digested genomic DNA to identify the 9.3 kb fragment of the wild-type allele and the 7.3 kb fragment of the targeted allele. **(C)** PCR-based genotyping confirmation of $Sox11^{loxP}$ mice using primers indicated in (A) distinguishes $Sox11^{loxP}$ and wild type allele. **(D)** *Sox11 in situ* hybridization of retina sections reveals a complete abolishment of *Sox11* expression in the retinas of $Sox11^{loxP/loxP}$*; Six3-Cre* mice. Scale bar: 100 μm.

FIGURE 3. **Abolishment of RGCs in *Sox4/11-null* retinas. (A-D, A'-D', F-I, F'-I')** Plastic sections of retinas isolated from 30 days old control, *Sox4-null* and *Sox11-null* mice were analyzed by hematoxylin and eosin (H&E) staining (A, A', F and F'), immunostaining of cryo-sections with anti-BRN3A (red) (B, B', G and G'), immunostaining of whole-mount retina with anti-BRN3B (green) (C, C', H and H') and SMI32 (green) (D, D', I and I'). **(K-N, K'-N")** Retinas of 14 days old control mice and *Sox4/11-null* mice were analyzed with H&E staining (K and K'), immunostaining of cryo-sections with anti-BRN3A (red) (L and L') and BRN3B (green) (M and M'). Immunostained sections with anti-BRN3A (red) and anti-BRN3B (green) were merged with DAPI staining pictures. **(E-E', J-J', O-O')** Ventral views of brains show thinner optic nerves (arrow heads) in *Sox4-null* and *Sox11-null* mice, and no optic nerves in *Sox4/11-null* mice. **(P and Q)** Quantification of BRN3B+ cells in the central region of the whole mounts shows a significant reduction of RGCs in *Sox4-null* or *Sox11-null* mice. All experiments were repeated at least three times. Error bars represent standard deviation (S.D.). * $P<0.01$. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer**;** ON, optical nerve. Scale bar: (A-D, A'-D', F-I, F'-I', K-N, K'-N') 50 μm; (E-E', J-J', O-O') 1 mm.

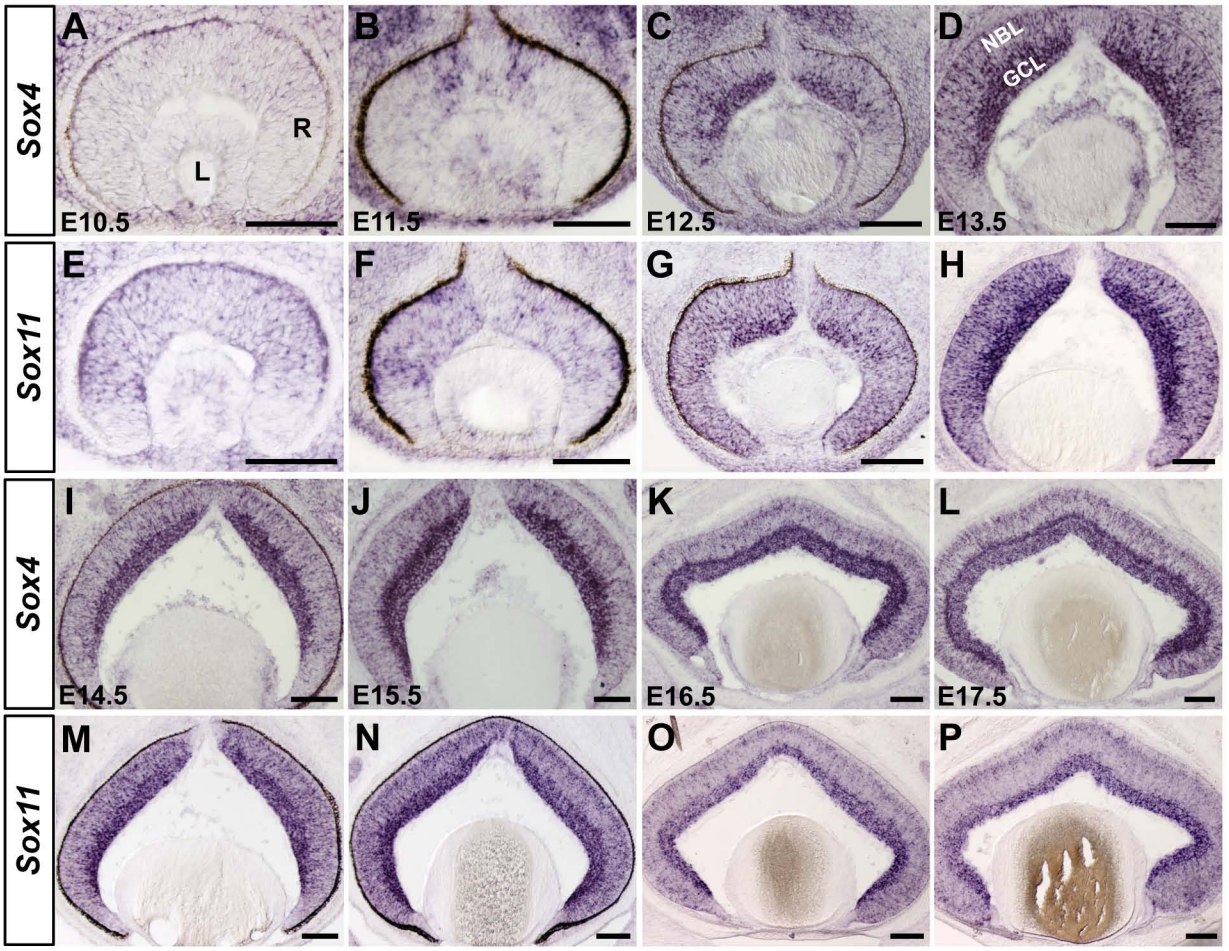
FIGURE 4. **Targeted disruption of *Sox4* and *Sox11* impairs the development of RGCs. (A-I, A'-I')** Cryo-sections of retinas from the control and mutant mice (*Sox4-null*, *Sox11-null*, and *Sox4/11-null*) at E12.5, E14.5, and E16.5 were immunolabeled with anti-BRN3B (green). **(J, K, L)** Quantification of RGCs in control and mutant mice at different embryonic stages. All experiments were repeated at least three times and error bars represent S.D. *$P<0.01$. GCL: ganglion, NBL: neuroblast layer. Scale bar: 50 μm.

FIGURE 5. **Increased number of apoptotic cells in *Sox4-null and Sox11-null* mice. (A-F, A'-F')** Immunostaining of cryo-sections of retinas from control and mutant (*Sox4-null, Sox11-null, Sox4/11-null*) mice at different embryonic stages with anti-CASPASE3 (green). **(G-H)** Quantification of number of apoptotic cells in control and mutant (*Sox4-null, Sox4-null,* and *Sox4/11-null*) retinas. All experiments were repeated three times and error bars are S.D. * $P<0.01$ Scale bar: 50 μm.

FIGURE 6. **Functional mechanisms of *Sox4* and *Sox11* in the development of RGCs. (A-H)** Expression of *Sox4* and *Sox11* in $Math5^{-/-}$ and $Brn3b^{-/-}$ retinas. *In situ* hybridization analysis of *Sox4* and *Sox11* expression in retinas of control and *Math5-null* mice at E13.5 (A-D). *In situ* hybridization analysis of *Sox4* and *Sox11* expression in retinas of control and *Brn3b-null* mice at E14.5 (E-H). **(I-K, I'-K')**

Expression of *Math5* in *Sox4-null*, *Sox11-null,* and *Sox4/11-null* retinas. *In situ* hybridization analysis of *Math5* expression in retinas of control and *mutant* mice at E12.5. **(L-N, L'-N')** Cryo-sections of *in vitro* cultured *Sox4* overexpressing retinal explants were immunostained with anti-GFP (green) and anti-BRN3B (red) as well as counter stained with DAPI (blue). **(O-Q, O'-Q')** Cryo-sections of *in vitro* cultured *Sox11* overexpressing retinal explants were immunostained with anti-GFP (green) and anti-BRN3B (red) as well as counter stained with DAPI (blue). **(R)** Quantification of RGCs in control and *Sox4* or *Sox11* overexpressing retina explants. **(S)** Schematic model of RGC development by *Sox4* and *Sox11* regulation. Solid lines, direct regulation of identified; broken lines, indirect or proposed regulation. All experiments were repeated at least three times and error bars are S.D. * $P<0.01$ Scale bar: (A-K, I'-K') 100 μm; (L-Q, L'-Q') 50 μm.

Figure 1


Sox4
Sox11
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A
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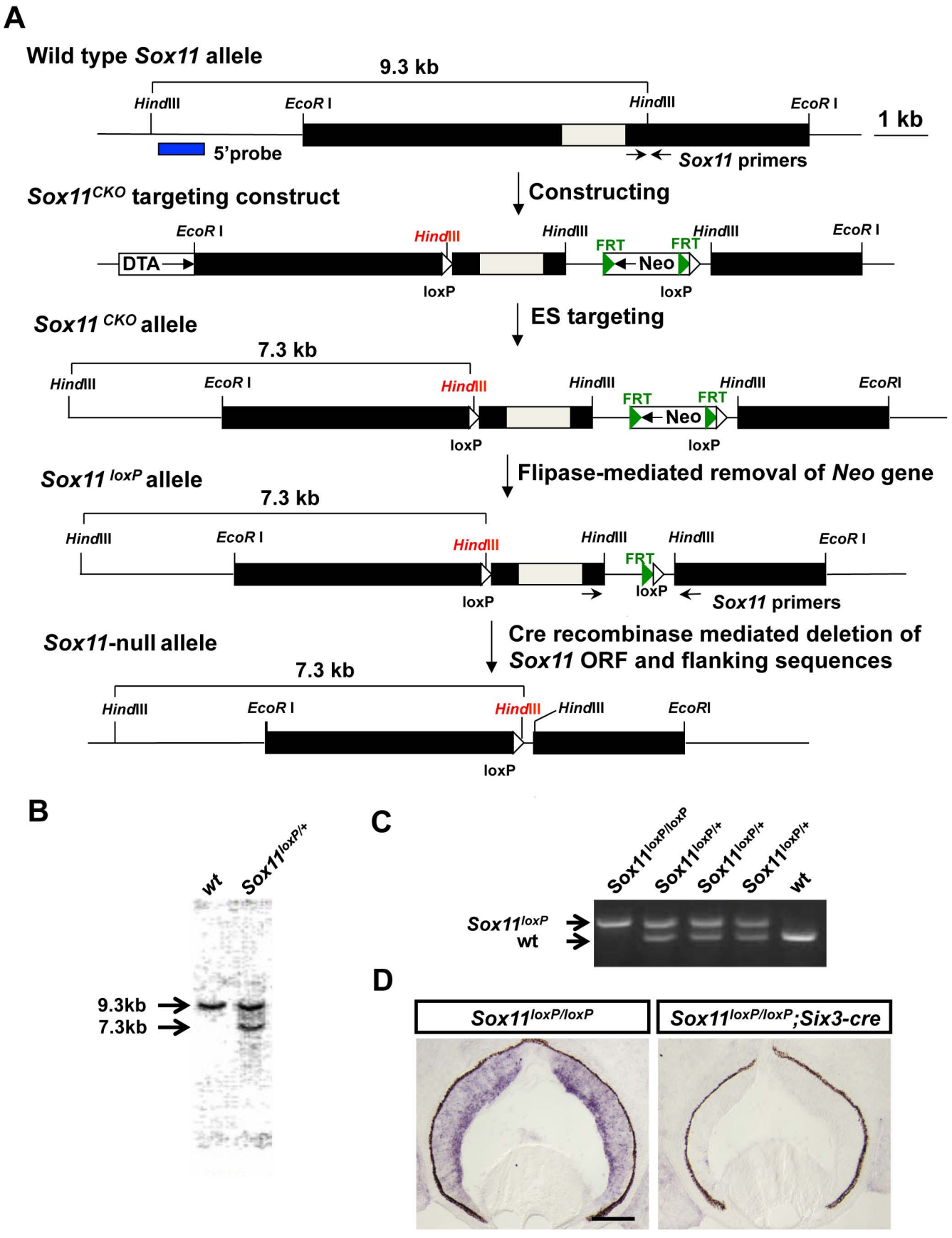
E17.5

Figure 2


A
Wild type Sox11 allele
9.3 kb
HindIII
EcoR I
HindIII
EcoR I
1 kb
5'probe
Sox11 primers
Sox11CKO targeting construct
Constructing
EcoR I
HindIII
HindIII
FRT
FRT
HindIII
EcoR I
DTA
Neo
loxP
loxP
Sox11 CKO allele
ES targeting
7.3 kb
HindIII
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FRT
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HindIII
EcoRI
Neo
loxP
loxP
Sox11 loxP allele
Flipase-mediated removal of Neo gene
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loxP
Sox11 primers
Sox11-null allele
Cre recombinase mediated deletion of Sox11 ORF and flanking sequences
7.3 kb
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EcoR I
HindIII
HindIII
EcoRI
loxP
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9.3kb
7.3kb
C
Sox11loxP/loxP
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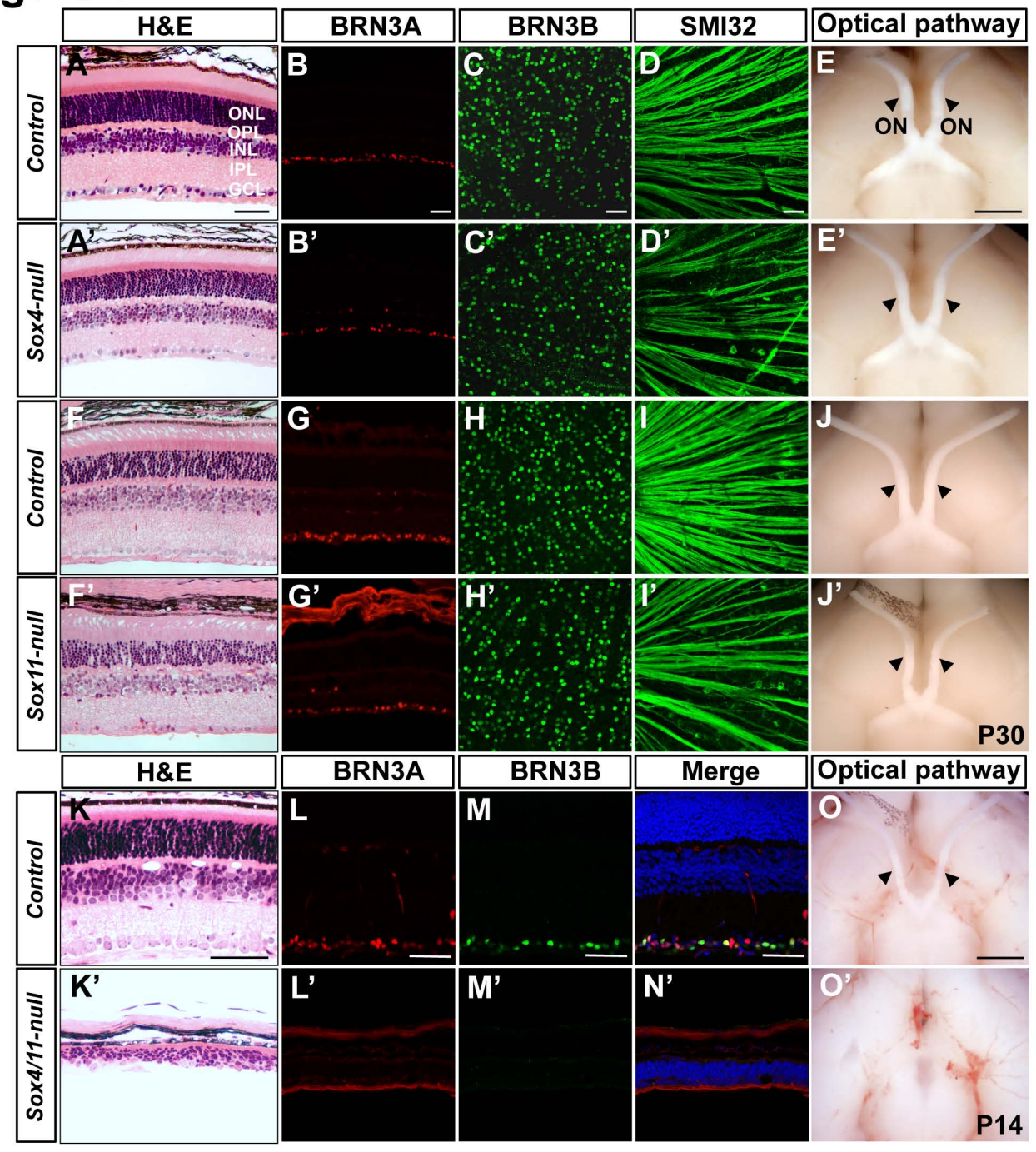
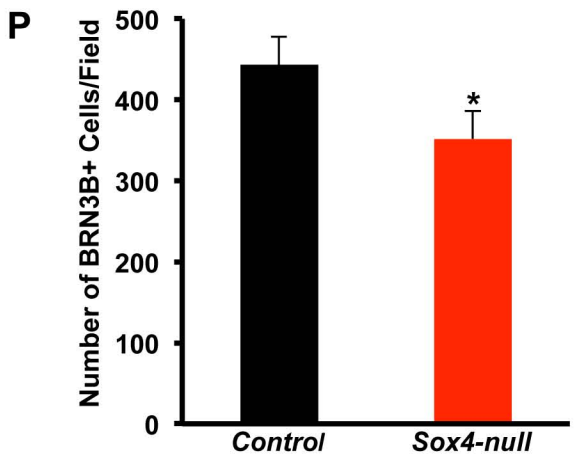
Sox11loxP/loxP;Six3-cre

Figure 3


H&E
BRN3A
BRN3B
SMI32
Optical pathway
Control
Sox4-null
Control
Sox11-null
A
B
C
D
E
ONL
OPL
INL
IPL
GCL
ON
ON
A'
B'
C'
D'
E'
F
G
H
I
J
F'
G'
H'
I'
J'
P30
H&E
BRN3A
BRN3B
Merge
Optical pathway
Control
Sox4/11-null
K
L
M
O
K'
L'
M'
N'
O'
P14


P
Number of BRN3B+ Cells/Field
500
400
300
200
100
0
*
Control
Sox4-null

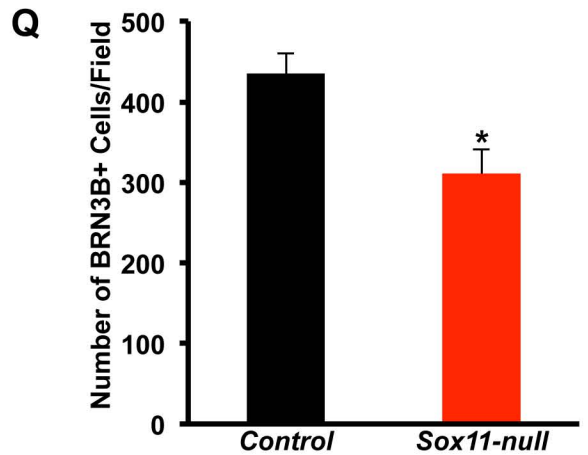
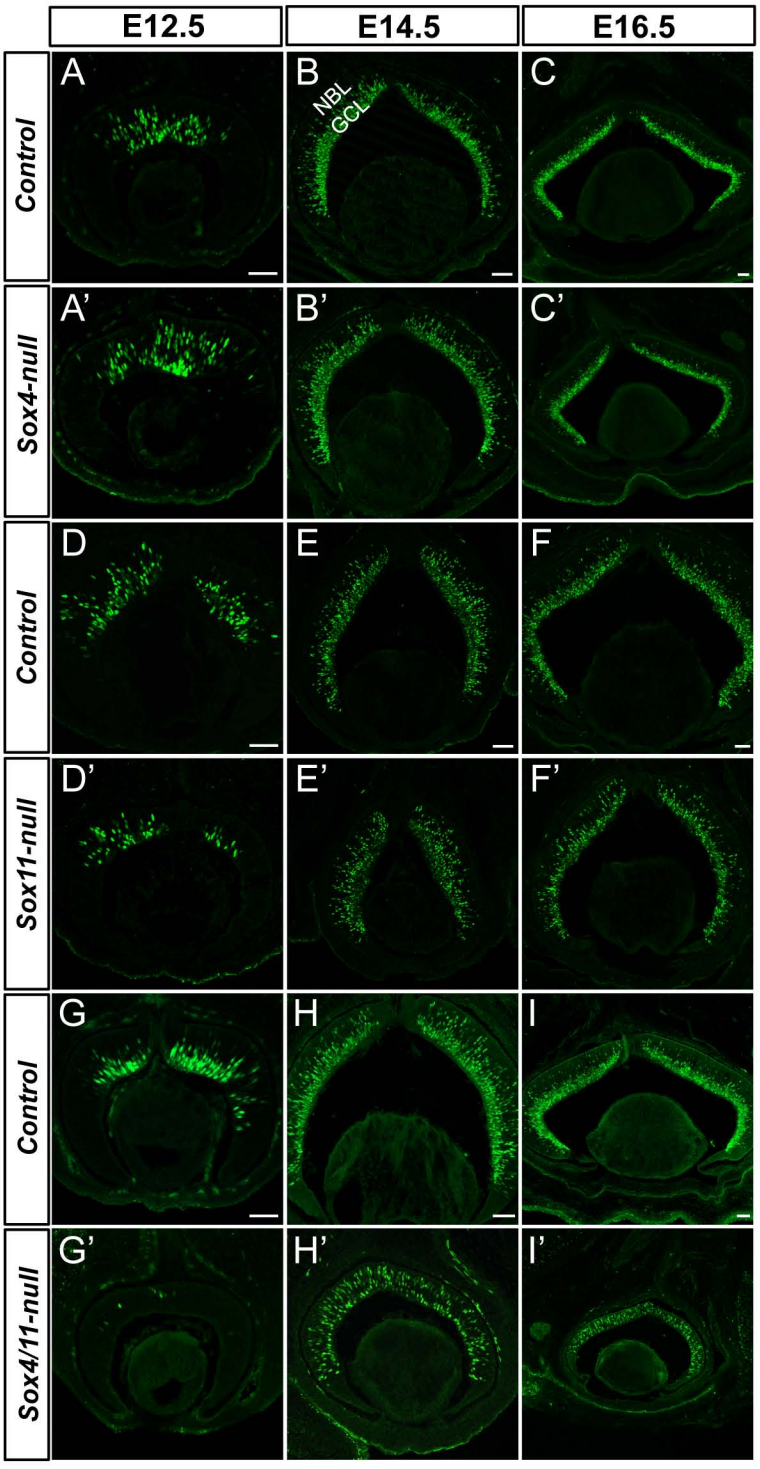
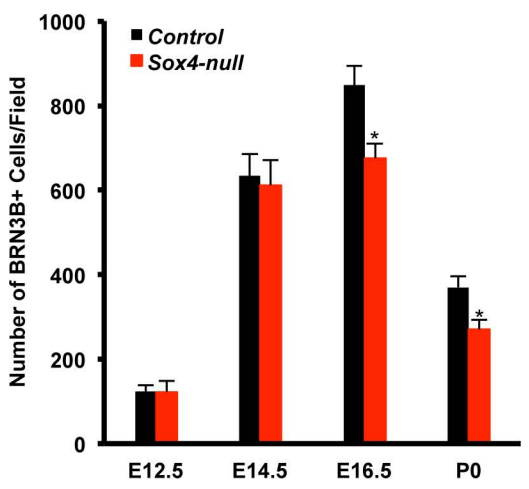
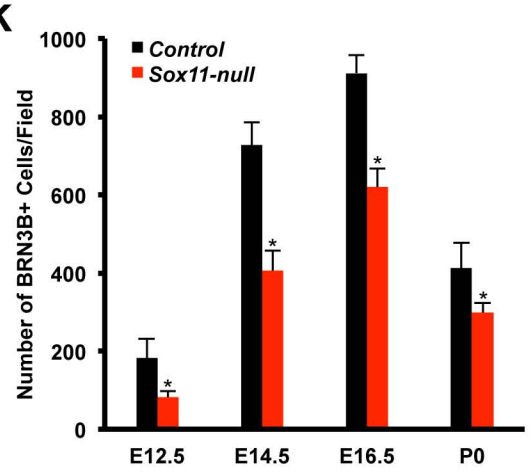

Q
Number of BRN3B+ Cells/Field
500
400
300
200
100
0
*
Control
Sox11-null

Figure 4


E12.5
E14.5
E16.5
Control
Sox4-null
Control
Sox11-null
Control
Sox4/11-null
A
B
C
A'
B'
C'
D
E
F
D'
E'
F'
G
H
I
G'
H'
I'
NBL
GCL


J
Control
Sox4-null
Number of BRN3B+ Cells/Field
1000
800
600
400
200
0
E12.5
E14.5
E16.5
P0


K
Control
Sox11-null
Number of BRN3B+ Cells/Field
1000
800
600
400
200
0
E12.5
E14.5
E16.5
P0

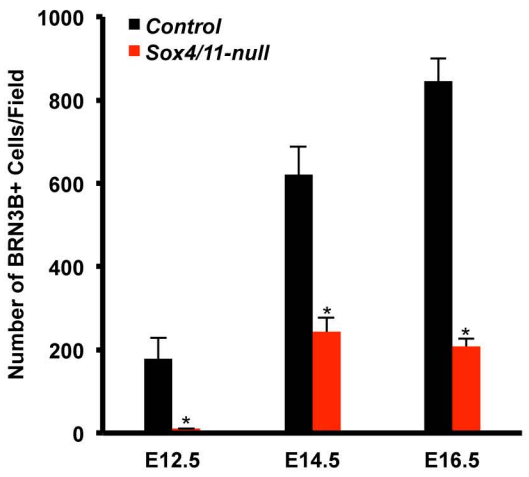
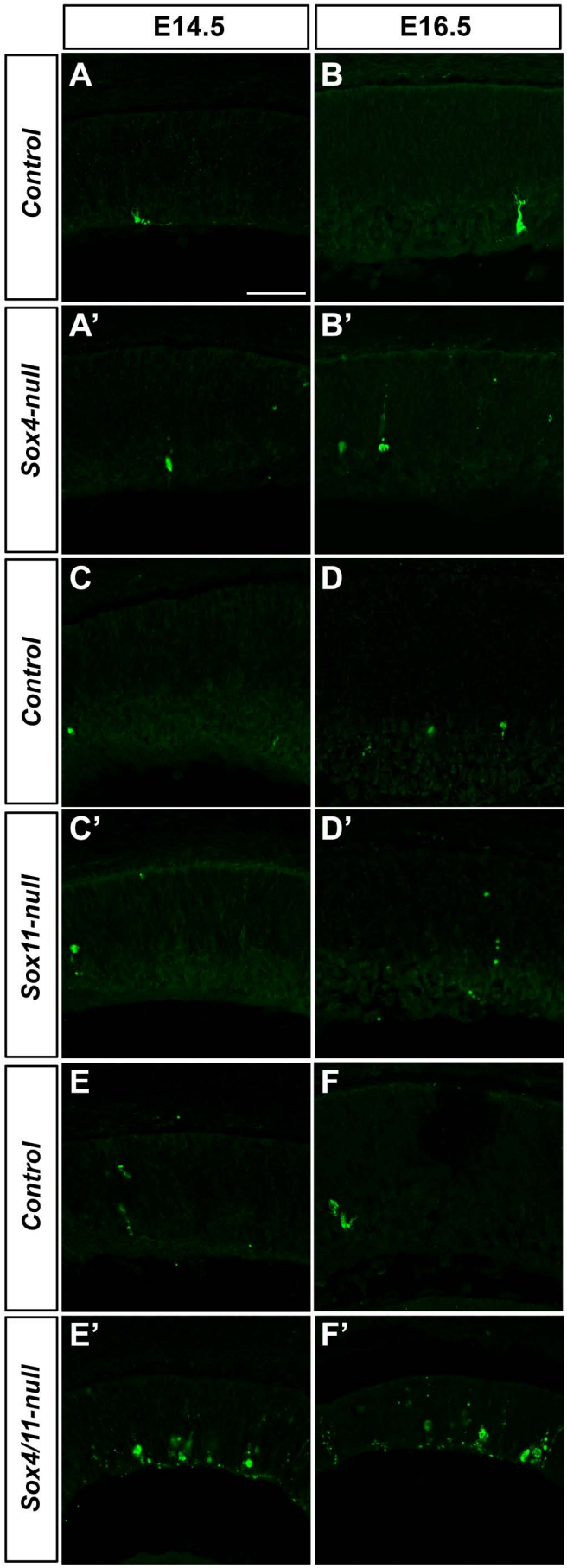
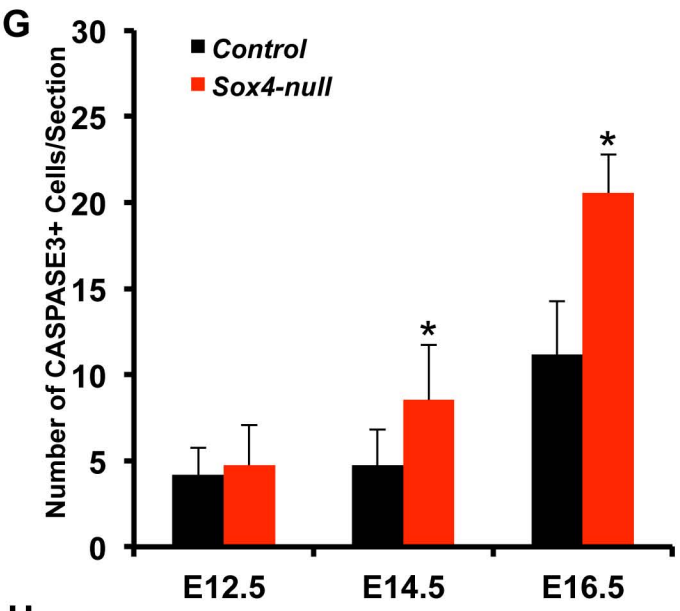
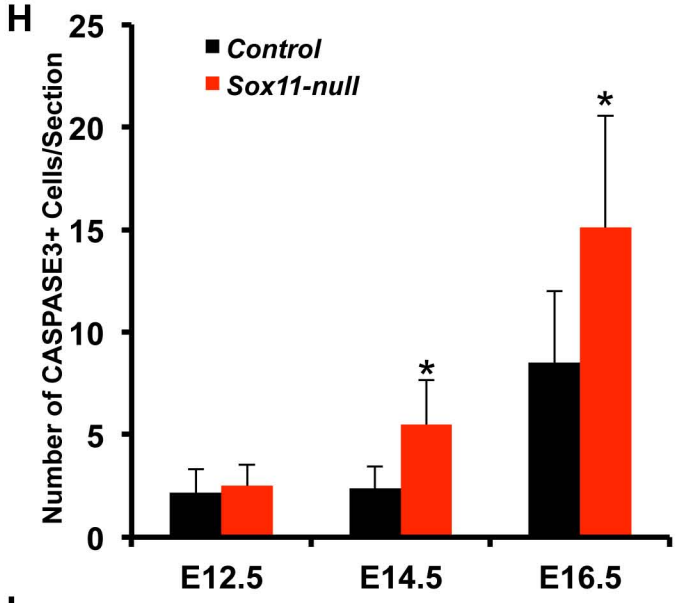

L
Control
Sox4/11-null
Number of BRN3B+ Cells/Field
1000
800
600
400
200
0
E12.5
E14.5
E16.5

Figure 5


E14.5
E16.5
Control
Sox4-null
Control
Sox11-null
Control
Sox4/11-null
A
B
A'
B'
C
D
C'
D'
E
F
E'
F'


G
Control
Sox4-null
Number of CASPASE3+ Cells/Section
0
5
10
15
20
25
30
E12.5
E14.5
E16.5
*
*


H
Control
Sox11-null
Number of CASPASE3+ Cells/Section
0
5
10
15
20
25
E12.5
E14.5
E16.5
*
*

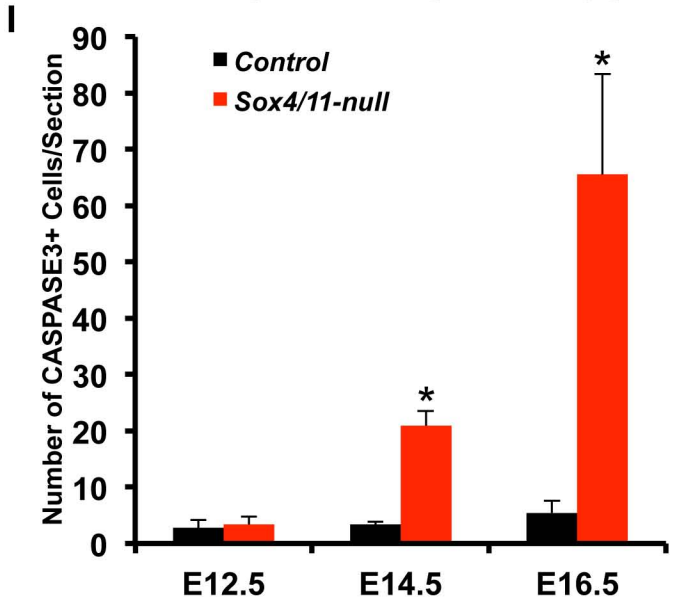
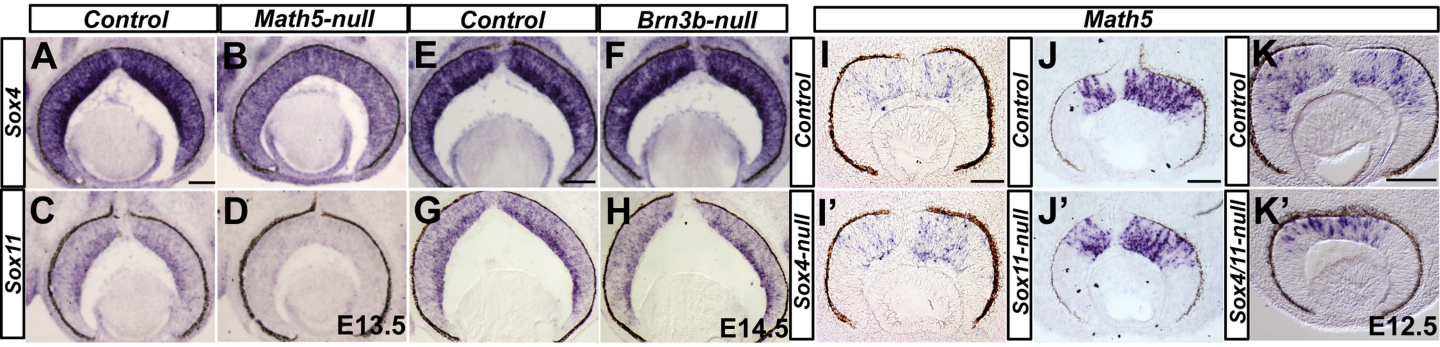
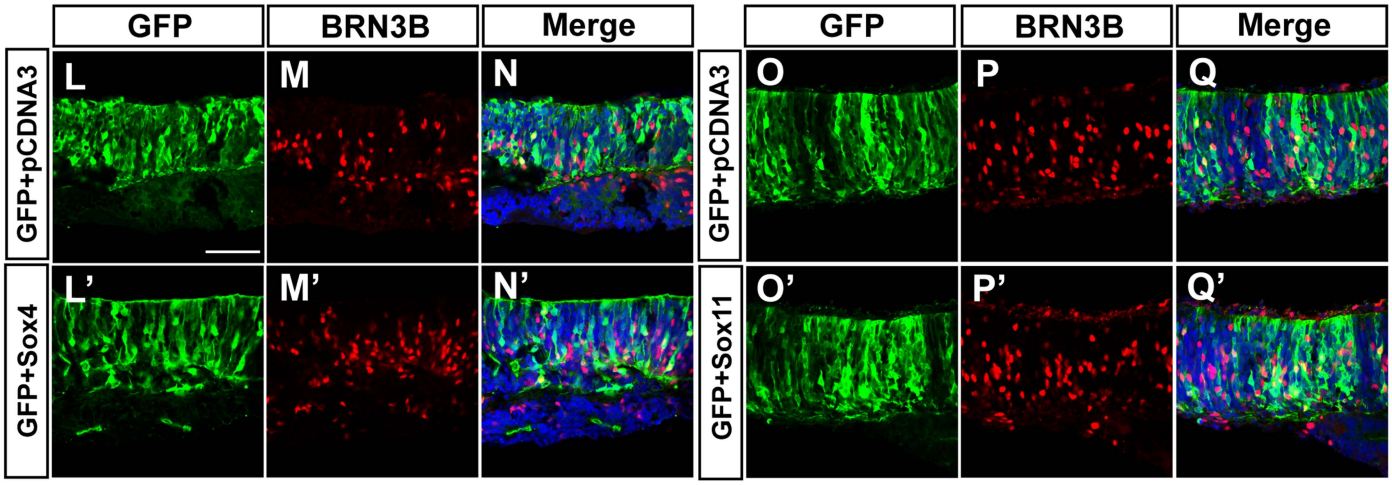
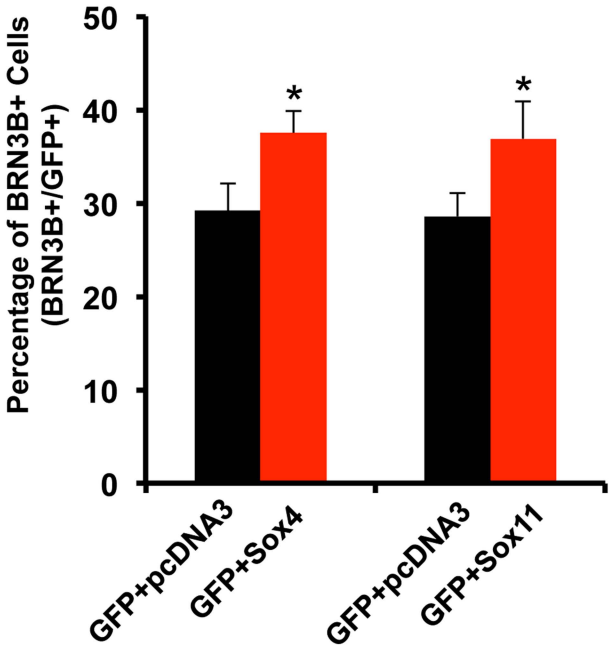

I
Control
Sox4/11-null
Number of CASPASE3+ Cells/Section
0
10
20
30
40
50
60
70
80
90
E12.5
E14.5
E16.5
*
*

Figure 6

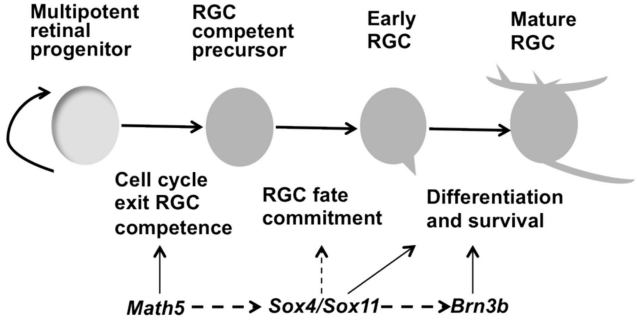

Control
Math5-null
Control
Brn3b-null
Math5
Sox4
Sox11
Control
Sox4-null
Sox11-null
Sox4/11-null
A
B
E
F
I
J
K
C
D
G
H
I'
J'
K'
E13.5
E14.5
E12.5


GFP
BRN3B
Merge
GFP+pCDNA3
GFP+Sox4
GFP+Sox11
L
M
N
O
P
Q
L'
M'
N'
O'
P'
Q'

R


Percentage of BRN3B+ Cells (BRN3B+/GFP+)
50
40
30
20
10
0
*
*
GFP+pcDNA3
GFP+Sox4
GFP+pcDNA3
GFP+Sox11

S


Multipotent retinal progenitor
RGC competent precursor
Early RGC
Mature RGC
Cell cycle exit RGC competence
RGC fate commitment
Differentiation and survival
Math5
Sox4/Sox11
Brn3b