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The early retinal progenitor-expressed gene *Sox11* regulates the timing of the differentiation of retinal cells

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SUMMARY

Sry-related HMG box (Sox) proteins, Sox11 and Sox4 are members of the SoxC subtype. We found that Sox11 was strongly expressed in early retinal progenitor cells and that Sox4 expression began around birth, when expression of Sox11 subsided. To analyze the roles of Sox11 and Sox4 in retinal development, we perturbed their expression patterns in retinal explant cultures. Overexpression of Sox11 and Sox4 in retinal progenitors resulted in similar phenotypes: an increased number of cone cells and dramatically decreased numbers of rod cells and Müller glia. Birth-date analysis showed that cone cells were produced at a later developmental stage than that in which cone genesis normally occurs. Sox11-knockout retinas showed delayed onset and progress of differentiation of subsets of retinal cells during the embryonic period. After birth, retinal differentiation took place relatively normally, probably because of the redundant activity of Sox4, which starts to be expressed around birth. Overexpression and loss-of-function analysis failed to provide any evidence that Sox11 and Sox4 directly regulate the transcription of genes crucial to the differentiation of subsets of retinal cells. However, histone H3 acetylation of some early proneural genes was reduced in knockout retina. Thus, Sox11 may create an epigenetic state that helps to establish the competency to differentiate. Taking our findings together, we propose that the sequential expression of Sox11 and Sox4 during retinogenesis leads to the fine adjustment of retinal differentiation by helping to establish the competency of retinal progenitors.

KEY WORDS: Sox family transcription factor, Retina, Progenitor cells, Mouse

INTRODUCTION

The vertebrate neural retina is organized into a laminar structure comprising six types of neurons and glial cells, Müller glia and astrocytes. In the mouse, these major retinal cell classes are generated from a common population of multipotent retinal progenitor cells between embryonic day (E) 11 and postnatal day (P) 10, in a conserved temporal order (Marquardt and Gruss, 2002). In vertebrates, retinal ganglion cells (RGCs) differentiate first, as a wave across the neuroepithelium of the optic cup. Ganglion cells, amacrine cells, cone photoreceptors and horizontal cells differentiate at relatively early stages primarily before birth, whereas bipolar cells and rod cells are mainly generated at later stages, after birth. It has been shown that both the progression of retinal neurogenesis and retinal cell fate specification or differentiation are controlled by intrinsic cues, such as transcription factors, as well as by extrinsic signals (Cepko, 1999; Harris, 1997).

To understand how this works, cell surface antigens are powerful tools for isolating specific subsets of retinal cells during development from cell mixtures without damaging the cells. This makes it possible to characterize their properties and identify genes that regulate their proliferation and differentiation. By screening retinal cells from mice at various developmental stages for their reactivity with over 150 different antibodies against various cell-surface antigens, we identified SSEA-1 (Fut4 – Mouse Genome Informatics) and Kit as early and late progenitor markers, respectively (Koso et al., 2006; Koso et al., 2007). SSEA-1 marks retinal progenitor cells in the peripheral region of the retina. In later stages of embryogenesis, SSEA-1 disappears, and Kit expression is observed in the retinal progenitor cells in the central region of retina. Using microarrays, we compared the gene expression patterns of regionally and temporally different subsets of retinal progenitor cells, SSEA-1-positive cells at E14 and Kit-positive cells at P1, and of differentiated cells, Kit-negative cells at P1. We found *Sox11* to be strongly expressed in SSEA-1-positive cells.

The Sry-related box (Sox) genes encode a group of transcription factors with a high mobility group (HMG)-type DNA-binding domain (Schepers et al., 2002). Based on their sequence homology, Sox proteins have been subdivided into groups A to J (Schepers et al., 2002). Mammalian SoxC proteins comprise Sox4, Sox11 and Sox12 (Schepers et al., 2002). All SoxC proteins are widely expressed during embryogenesis in neuronal progenitors and in mesenchymal cells in many developing organs (Dy et al., 2008; Hoser et al., 2008). The functions of SoxC genes as regulators of cell fate, proliferation and survival in major physiological and pathological processes have been reported in many organ lineages. However, the expression and function of SoxC in the developing retina is unknown. Sox11 knockout (KO) mice has been reported (Sock et al., 2004), with the eyes showing microphthalmia and anterior segment dysgenesis such as Peter's anomaly (Wurm et al., 2008). By gain-of-function analysis of Sox11 and Sox4, as well as a detailed examination of Sox11-KO retinas, we are able to describe the roles of Sox11 and Sox4 in retinal development.

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MATERIALS AND METHODS

Mice and reagents

Sox11-knockout (Sox11-KO; $Sox11^{LacZ/LacZ}$) embryos were obtained from timed mating of $Sox11^{LacZ/+}$ (Sox11-LacZ/+) mice (Sock et al., 2004). ICR mice were obtained from Japan SLC. All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo and conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research. Microarray analysis was carried out using Affymetrix GeneChip Mouse Genome 430 2.0 using total RNA from retinas of Sox11-KO at E18 and littermate. Microarray data are available in the GEO database (Accession Number GS43089). shRNA plasmid for Sox4 (pGFP-V-RS shRNA) was purchased from Origene, and efficiency was examined by RT-PCR in NIH3T3 cells. The target sequence was 5′-CGGCTGCATCGTTCTCTCCAGAGCAAGCT-3′. Retinal explants and retroviral infection were performed as described elsewhere (Tabata et al., 2004). *In vitro* electroporation was carried out as described (Iida et al., 2011). The electroporated retinas were cultured at 34°C on a chamber filter (Millicell).

DNA construction

Full-length cDNAs encoding mouse Sox4, and Sox11 open reading frames (ORFs) were isolated by reverse transcription (RT)-PCR using mouse retinal RNA and then cloned into pGEM-T-Easy vector (Promega). Sox4 and Sox11 fragments were subcloned into pMX-IRES-EGFP retrovirus vector using *Bam*HI/*Xho*I (Sox11) and *Eco*RI (Sox4) sites and pCAG vector using *Eco*RI (Sox11) and *Eco*RV/*Xho*I (Sox4).

RT-PCR

Total RNA was purified from mouse retinas using RNeasy Plus Micro (QIAGEN), and cDNA was synthesized using Superscript II (Invitrogen-Gibco). For semi-quantitative PCR, Blend Taq Plus (TOYOBO) was used, and bands were visualized with ethidium bromide. Quantitative PCR (qPCR) was carried out using the SYBR Green-based method using the Roche Light Cycler 1.5 apparatus and analyzed by the Second Derivative Maximum Method for quantification (Roche Diagnostics). The sequences of PCR primers are listed in supplementary material Table S2.

In situ hybridization of Sox11 and Sox4

In situ hybridization was performed according to a standard protocol previously described (Koso et al., 2008) using digoxigenin-labeled RNA probes. As the coding regions of Sox11 and Sox4 have high homology in the 5′ half, we used the 3′ half and the 3′ untranslated region of cDNA using a common *Apa*I site in the middle of the Sox11- and Sox4-coding regions.

Immunostaining

Immunostaining of sections was carried out as described previously (Tabata et al., 2004). The first antibodies were visualized by using appropriate Alexa Fluor-conjugated second antibodies (Molecular Probes). Samples were mounted in VectaShield (Vector Laboratories) and analyzed using a Zeiss Axio Vision 4.6 microscope. The primary and secondary antibodies are listed in supplementary material Table S1.

Fluorescence-activated cell sorting (FACS)

Retinas electroporated with plasmids containing EGFP were digested with trypsin (0.25%) at 37°C for 10 minutes. PBS containing FCS (20%) and DNase I (0.2%) was added, and the cells were mechanically dissociated into a single-cell suspension by gentle pipetting. Sorting was carried out using a MoFlo (DakoCytomation).

Chromatin immunoprecipitation (ChIP) assay

Mouse retinas crosslinked with 1% formaldehyde were suspended in 1% SDS lysis buffer and sonicated to shear genomic chromatin. The lysate was incubated for 1 day with the antibody-bound Dynabeads-Protein G (Invitrogen). Eluated immune complex was incubated at 65°C overnight and proteins were eliminated by proteinase K (Wako). DNA was purified with a QIAquick PCR purification kit (QIAGEN). Real-time PCR was carried out using a Roche Light Cycler 1.5 apparatus. The sequences of PCR primers are listed in supplementary material Table S2. The abundance of target genome DNA was normalized relative to that of input. For all ChIP experiments, independent experiments were carried out at least twice, and essentially the same results were obtained. One representative set of data are shown. Control IgG experiments gave only negligible values.

BrdU labeling and birth-date analysis

For pulse labeling with BrdU to detect S-phase RPCs, 100 μg of bromodeoxyuridine (BrdU; Sigma-Aldrich) per gram of body weight was injected intraperitoneally into pregnant females 1 hour before being euthenized. Embryonal heads were fixed in 4% PFA and frozen sectioned. For retinal explants, after 3 days of culture, BrdU was mixed into medium at a final concentration of 1.5 μg/ml at 24 hours before fixation. For birth-date analysis, pregnant Sox11-LacZ/+ mice were intraperitoneally injected with BrdU (100 μg per gram of body weight) at 11, 13 or 15 days of pregnancy, and sacrificed at day E18. Retinas were frozen sectioned and immunostained. For Sox11 overexpression, pMX-Sox11-EGFP was electroporated *in vitro* into isolated retina from normal embryos at E14 and cultured as explants. BrdU was present in the first, second, third, fourth, fifth and sixth 24 hours of explant culture, and retina was harvested at the 14th day and frozen sectioned. Sections were immunostained with antibodies as indicated.

RESULTS

Sox11 is expressed in the retina during early developmental stages and Sox4 at a later stage

We previously found that SSEA-1 and Kit mark subsets of retinal progenitor cells in early and late embryonic stages, respectively (Koso et al., 2006; Koso et al., 2007). Using microarrays, we then searched for genes that are specifically expressed in retinal progenitor cells. We found that Sox11 was more strongly expressed in SSEA-1-positive cells than in Kit-positive cells and that Sox4 showed the reverse pattern (supplementary material Table S3). Other microarray analyses comparing the gene expression pattern of E15 retinas with that of explant retinas cultured for 5 days showed that expression of Sox11 decreased as retinal development proceeded; by contrast, expression of Sox4 increased (supplementary material Table S4). Another member of the SoxC group, Sox12 (Bowles et al., 2000), showed only negligible expression in all subsets/developmental stages examined (supplementary material Tables S3, S4). We then examined the time course of Sox11 and Sox4 expression in more detail by semi-quantitative RT-PCR. Sox11 was strongly expressed in E14 and E16 retinas, with the expression level becoming slightly weaker at E18 and decreasing sharply after birth (Fig. 1A). Sox4 was only weakly expressed in E14 retinas; its expression subsequently increased gradually, peaking at E18 (Fig. 1A). Expression continued until P12 and became faint by around P15.

We next examined the spatial patterns of Sox11 and Sox4 expression by *in situ* hybridization (Fig. 1B). Sox11 was expressed throughout the retina at E11 and became stronger in the inner half of the retina at E13. At E17, strong expression of Sox11 was observed in the ganglion cell layer (GCL). From P3 onwards, we could not detect Sox11 (Fig. 1B). We observed a faint Sox4 signal in the central region of the retina at E11; at E13, expression was detected in the GCL (Fig. 1B). At E17, a pattern similar to that at E13 was noted; between P3 and the adult stage, expression of Sox4 occurred in the GCL and the inner nuclear layer (INL) (Fig. 1B).

Sox11 is expressed in proliferating cells and in early retinal cells

We next examined the expression of Sox11 in detail by immunostaining *lacZ* (β-galactosidase) in Sox11-*lacZ* mice carrying a *lacZ* gene in the Sox11 locus (Sock et al., 2004). First,

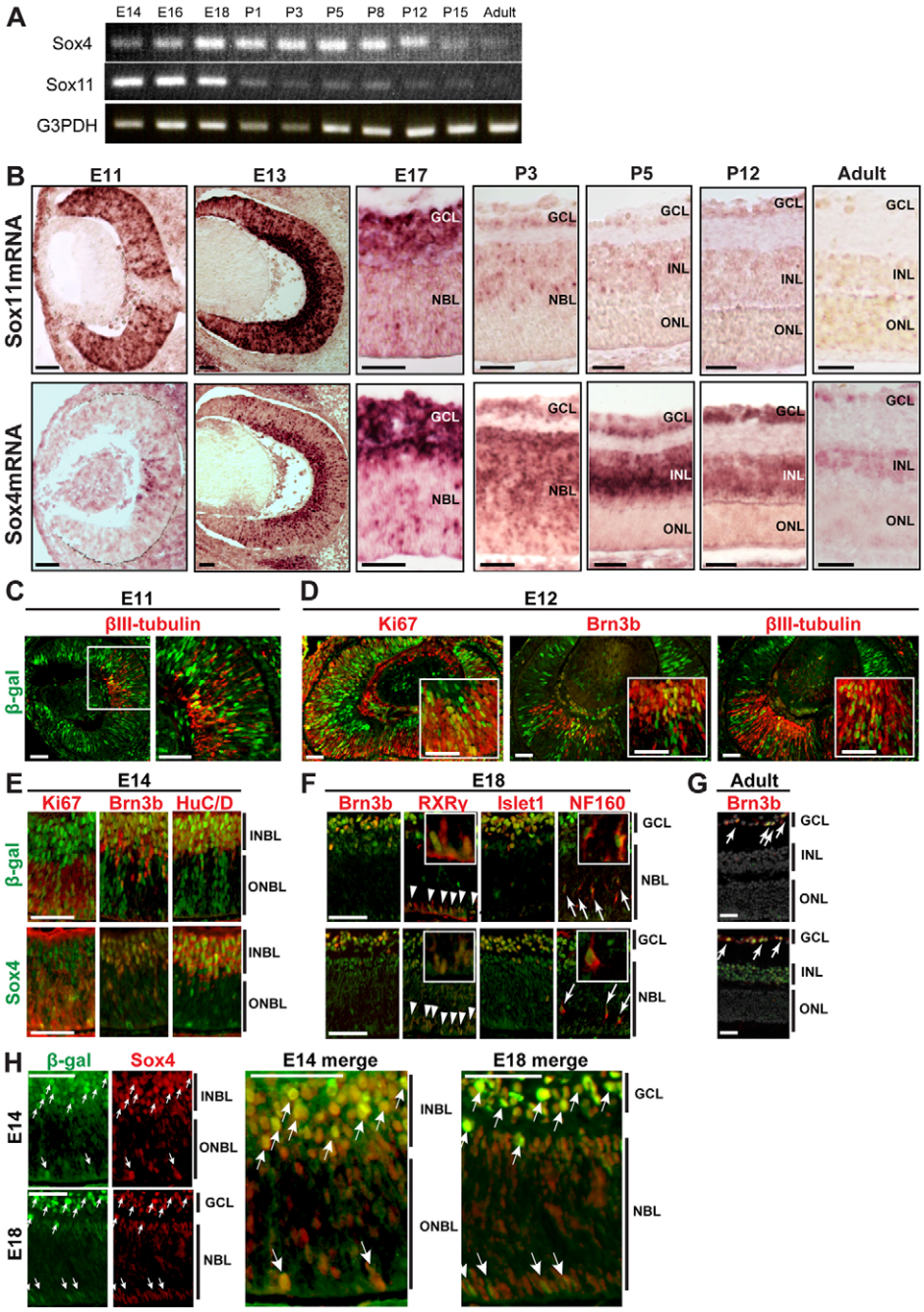

Fig. 1. Expression of Sox4 and Sox11 during retinal development.
(**A**) Expression of Sox11 and Sox4 mRNA in retinas was examined at various developmental stages, as indicated by semi-quantitative RT-PCR of Sox4 and Sox11. G3PDH was used as a control. (**B**) *In situ* hybridization of Sox11 and Sox4 was carried out using retinal sections at various stages as indicated. (**C-G**) Expression of Sox11 or Sox4 and various retinal markers at indicated developmental stages. Sox11 expression was visualized by immunostaining using anti β-galactosidase antibody on frozen retinal sections from Sox11-*lacZ*/+ mice. Sox4 protein expression was shown by immunostaining of Sox4 antibody. (**H**) Expression of Sox11 and Sox4 were shown by co-immunostaining of β-galactosidase and Sox4. Panel insets are enlarged views of areas indicated by white squares in C,D,F. Arrowheads and arrows indicate positive cells (F-H). Antibodies used to visualize retinal subsets were βIII-tubulin (neuronal marker), Ki67 (progenitor marker), Brn3b (ganglion cell marker), HuC/D (ganglion and amacrine cell marker), RXRγ (cone photoreceptor marker), Islet1 (ganglion and amacrine cell marker) and NF160 (horizontal cell marker). INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer; GCL, ganglion cell layer; NBL, neuroblastic layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 50 μm. Nuclei are visualized using DAPI (blue).

we confirmed that the gross structure of the retina and the expression patterns of various markers of retinal cell subtypes in mature retinas of Sox11-*lacZ* heterozygous (*lacZ*/+) mice were indistinguishable from those in wild-type retinas (data not shown). Next, we confirmed that the *lacZ* expression pattern was similar to that obtained by *in situ* hybridization of Sox11 (data not shown, Fig. 1C-F). We next conducted double staining of *lacZ* and retinal subtype markers using retinal sections derived from Sox11-*lacZ*/+ mice. At E11, βIII-tubulin started to be expressed, and some Sox11-expressing cells in the central region exhibited βIII-tubulin signals (Fig. 1C). At E12, Sox11 was mainly expressed in Ki67-positive retinal progenitor cells. βIII-tubulin signals became stronger, and a subset of the βIII-tubulin-positive cells expressed Sox11 (Fig. 1D). At that time, Brn3b (Pou4f2 – Mouse Genome Informatics), a marker of retinal ganglion cells, began to be expressed; some Brn3b-positive cells expressed Sox11 (Fig. 1D). At E14, Sox11 and Sox4 signals in the innermost region merged with those for Brn3b and HuC/D, the latter of which labels ganglion and amacrine cells (Fig. 1E). Diffuse and weak expression in the outer two-thirds of the region coincided with Ki67 (Fig. 1E). At E18, strong Sox11 and Sox4 expression in the GCL coincided with the expression of Brn3b and Islet1, the latter of which is expressed in ganglion and amacrine cells (Fig. 1F). Most outer cells were positive for RXRγ and most cells in the middle part were positive for NF160, suggesting that they are cone and horizontal cells, respectively (Fig. 1F, arrowheads, arrows). In adults, weak expression of β-gal was observed in the GCL (Fig. 1G, arrow). We next examined whether Sox11 and Sox4 were co-expressed during

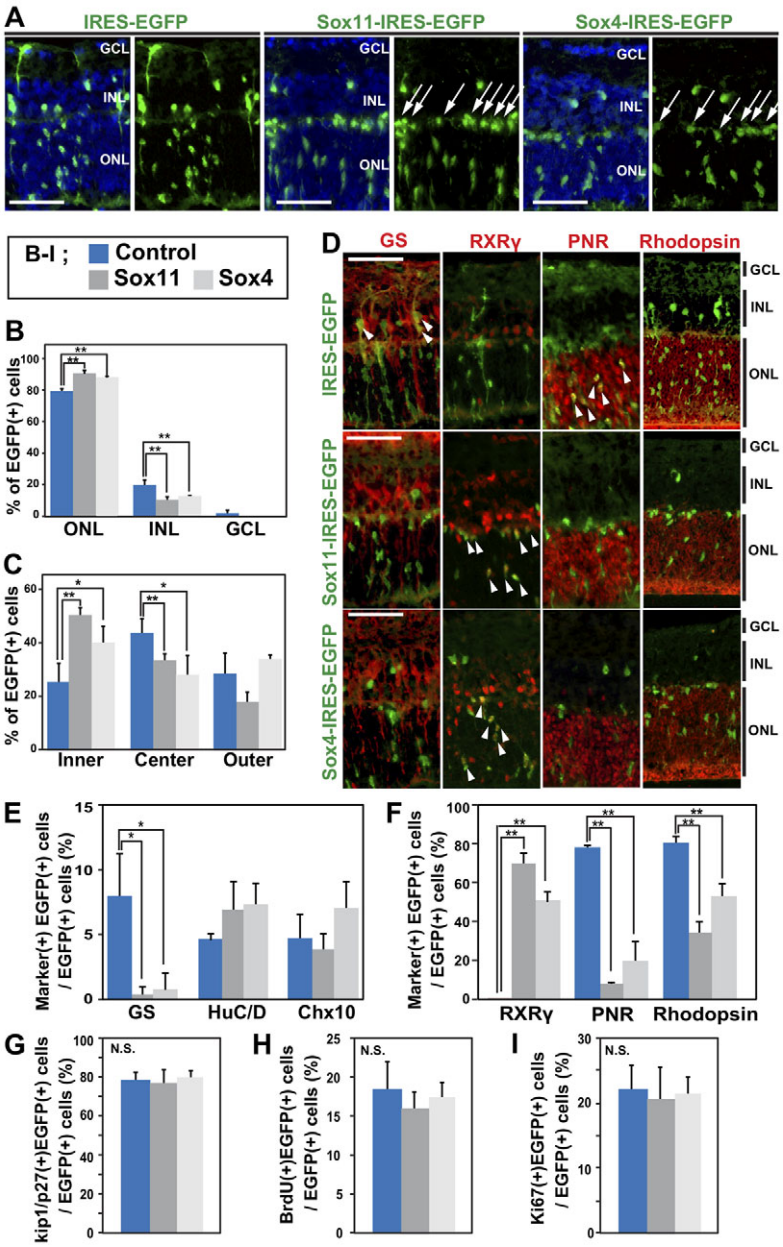

Fig. 2. Gain-of-function analysis of Sox11 and Sox4 resulted in enhancement of cone and suppression of rod and Müller glia retinal subtypes. (**A-F**) Retrovirus encoding Sox11-IRES-EGFP, Sox4-IRES-EGFP or control EGFP was transduced into retinal explants at E17 and cultured for 2 weeks. Frozen sections were made and stained with antibody against EGFP (A-C) or with EGFP in combination with various retinal subtype-specific markers (D-F). Arrowheads indicate double-positive cells (D). Population of EGFP-positive cells in retinal sublayers (ONL, INL, GCL) is shown in B. The ONL was divided horizontally into three regions and EGFP-positive cells in each subregion are shown in C. (F) Population of EGFP and marker double-positive cells out of EGFP single-positive cells is shown. (**G-I**) Proliferation-related indicators were examined 72 hours after retrovirus infection. In H, BrdU was present during the last 24 hours of culture. The average of three independent experiments with standard deviation is shown. $**P<0.01$, $*P<0.05$, calculated using Student's *t*-test. Scale bars: 50 μm. N.S., not significant.

retinal development by immunostaining Sox4 and β-gal. Sox4 showed an expression pattern similar to that produced by *in situ* hybridization (data not shown, Fig. 1E-G), and co-immunostaining of Sox11-*lacZ*/+ retinas with β-gal showed that Sox11 and Sox4 were co-expressed in cells in the INL at E14 and cells of the INL and GCL at E18 (Fig. 1H, arrows).

Gain-of-function analysis of Sox11 and Sox4 revealed altered retinal differentiation

To delineate the functions of Sox11 and Sox4 in retinal development, we conducted a gain-of-function analysis by retrovirus-mediated gene transfer into retinal explant cultures (Tabata et al., 2004). A retrovirus encoding Sox11-IRES-EGFP or Sox4-IRES-EGFP was used to infect retinal explants prepared from embryos at E17, and the sublayer distributions of Sox11- and Sox4-overexpressing cells were examined after 2 weeks of culture. The number of Sox11-overexpressing cells in the outer nuclear layer (ONL) increased slightly, and that in the INL decreased (Fig. 2A,B). Although control virus-infected cells were distributed throughout the ONL (Fig. 2A,C), Sox11-overexpressing cells in the ONL tended to be localized more basally, and many cells aligned with the inner edge of the ONL (Fig. 2A, arrows). Comparable results for all features were obtained for Sox4 (Fig. 2A,C).

We next examined the retinal subtypes of Sox11- and Sox4-overexpressing cells by immunostaining various markers specific for retinal cells subtypes. Müller glia marked by GS were nearly absent in Sox11- and Sox4-expressing cells (Fig. 2D,E). The populations of HuC/D- and/or Chx10-positive amacrine, horizontal and bipolar cells were slightly affected by Sox11 and Sox4, but the difference was not statistically significant (Fig. 2E). In explant cultures, cone cells usually localized at the border of the ONL and the outer plexiform layer (OPL). Most cells, including those localized immediately proximal to the OPL, expressed the cone photoreceptor marker RXRγ, suggesting increased numbers of cone cells in Sox11- and Sox4-overexpressing cultures (Fig. 2D,F).

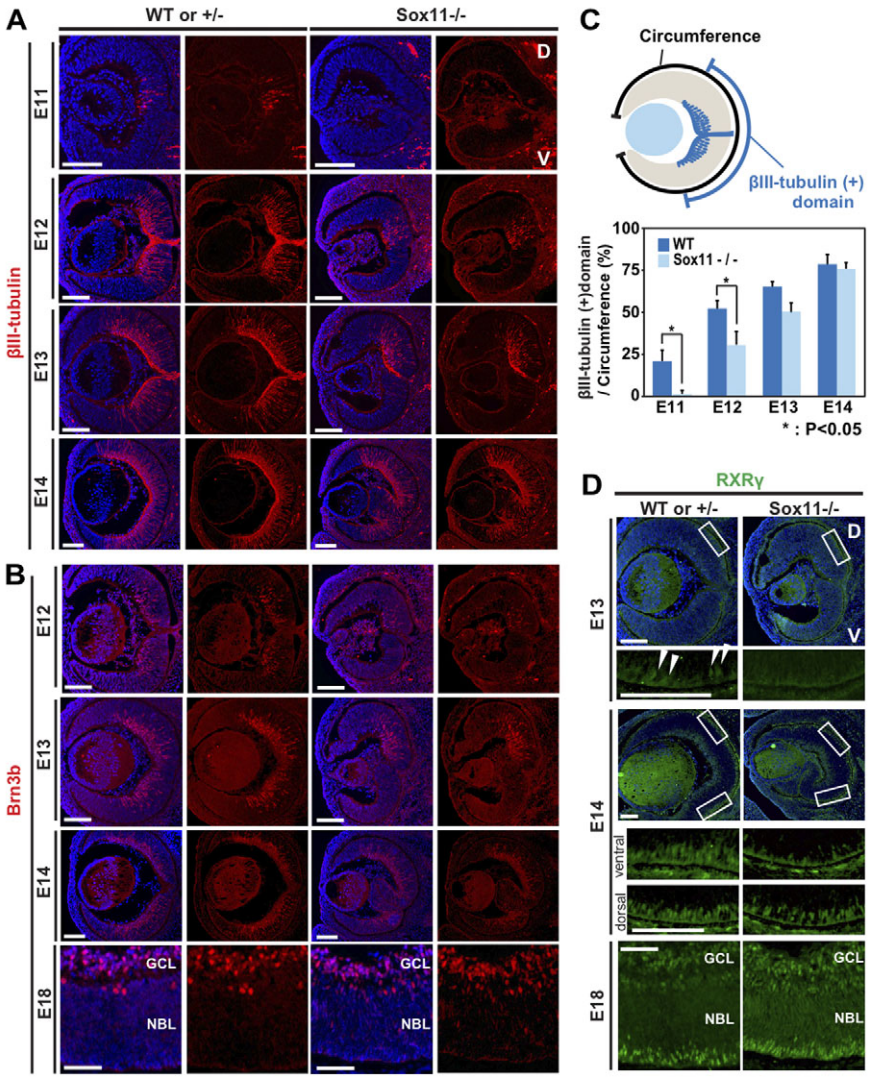

Fig. 3. Sox11 loss of function delayed the differentiation of subsets of retinal cells. (**A**,**B**,**D**) Neurogenesis and differentiation of retinal neurons in Sox11-KO retinas were analyzed. Transverse sections of embryos of littermates or Sox11-KO at E11-E18, or sections of isolated retinas at E18, were immunostained with indicated antibodies. Antibodies used were βIII-tubulin, Brn3b and RXRγ. Nuclei were visualized by DAPI staining. Scale bars: 100 μm. Lower panels of E13 and E14 in D are enlarged views of the area indicated by white squares of the upper one. (**C**) Measurement scheme for retinal circumference and βIII-tubulin expression domain widths. The percentage of βIII-tubulin-positive domain per total circumference of retina is shown. The average of three independent experiments with standard deviation is shown. **P*<0.05, calculated using Student's *t*-test..

However, these cells expressed neither cone arrestin 4 nor M-opsin (supplementary material Fig. S1), suggesting that final maturation did not occur with these cells. By contrast, expression of Sox4 or Sox11 dramatically suppressed rod cell differentiation (Fig. 2F, PNR, rhodopsin), suggesting that the increase in the number of cones occurs at the expense of rod cells. All criteria showed similar activities for Sox11 and Sox4, suggesting that Sox11 and Sox4 are redundant in terms of retinal cell differentiation.

The Notch signaling pathway affects the differentiation of Müller glia and cone photoreceptors in positive and negative ways (Furukawa et al., 1997; Hojo et al., 2000; Jadhav et al., 2006; Yaron et al., 2006; Riesenberg et al., 2009). We examined whether Sox4 and Sox11 affect Notch signaling by analyzing RBP-jκ-luciferase, a reporter for Notch signaling (Minoguchi et al., 1997). No effect was observed (supplementary material Fig. S2).

As altered timing of exit from the cell cycle often results in perturbation of differentiation, we next examined the effects of Sox11 and Sox4 on retinal cell proliferation by assessing BrdU incorporation and the expression of Kip1/p27 and Ki67. None of these parameters showed significant differences in Sox11- or Sox4-overexpressing cells compared with control cells (Fig. 2G-I), suggesting that Sox11 and Sox4 may not play important roles in the proliferation of retinal cells.

Loss of Sox11 function in retinas delayed the initiation of neurogenesis and differentiation of ganglion and cone cell subtypes

To analyze the *in vivo* role of Sox11 in retinal development, we examined retinas from Sox11-*lacZ*/*lacZ* homozygous null (Sox11-KO) mice (Sock et al., 2004). Analysis of the gross morphology of the eyes in Sox11-KO mice at E18 showed microphthalmia and anterior segment dysgenesis, as previously reported (Wurm et al., 2008). We next examined the retinal phenotype in more detail by immunostaining with antibodies specific for markers of retinal cell subtypes. The first retinal neuron ganglion cells appear in the dorso-central retina, and neurogenesis spreads peripherally (Hufnagel et al., 2010). At E11, βIII-tubulin and Brn3b were expressed in the dorso-central retina, but were not detected in the Sox11-KO retina (Fig. 3A). At E12, ganglion cells started to differentiate in the control retina. By contrast, only a few Brn3b- or βIII-tubulin-positive cells were observed in the Sox11-KO retina (Fig. 3A,B). At E13, the areas in which βIII-tubulin and Brn3b were expressed expanded, but only the central part of the retina expressed these proteins in Sox11-KO retinas. However, at E14, the βIII-tubulin- and Brn3b-positive areas in the Sox11-KO retina started to expand (Fig. 3A,B). Quantification of the peripheral spread of

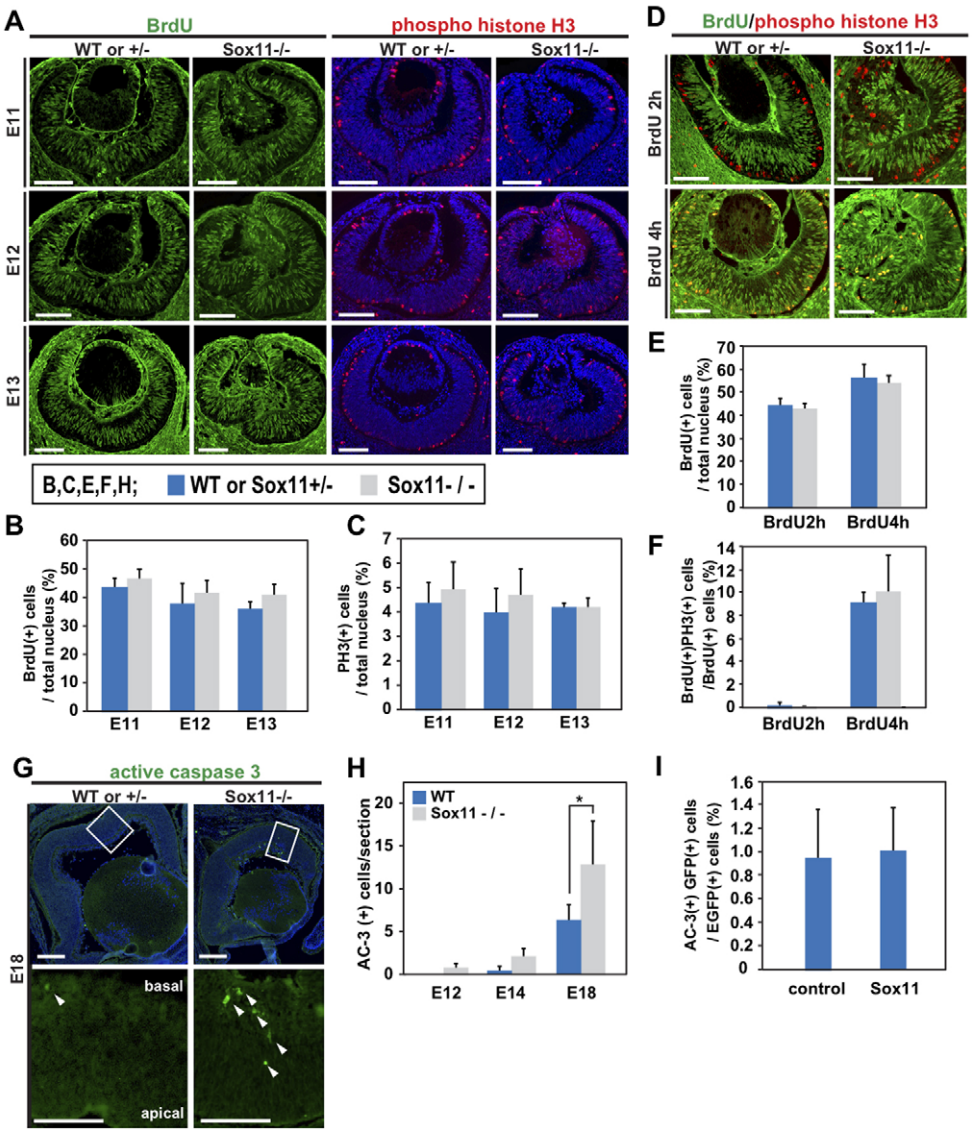

Fig. 4. Sox 11 loss of function did not affect retinal proliferation. (**A**-**C**) Proliferation of Sox11-KO retina was examined by BrdU incorporation and phospho-histone H3 (PH3) expression. For BrdU incorporation, pulse labeling of BrdU was conducted by injection of BrdU into pregnant females intraperitoneally 2 hours before being euthenized. Embryos were sacrificed at indicated stages, and frozen head sections were used to examine BrdU incorporation and PH3 by immunostaining (A-C). (**D-F**) BrdU labeling was carried out for 2 or 4 hours followed by immunostaining of BrdU and PH3 (D). Number of BrdU-positive cells (E) and BrdU and PH3 double-positive cells (F) was examined. (**G**,**H**) Apoptotic cells were examined by immunostaining active caspase 3 (AC-3)-positive cells at E12, E14 and E18. Arrowheads indicate marker-positive cells (G). (**I**) Apoptotic cells were examined in Sox11-overexpressed retina. Retrovirus encoding Sox11 was infected to E14 retina, and after 3 days, apoptotic cells were examined by immunostaining of active caspase 3. Nucleus was visualized with DAPI. In B,C,E,F,H,I, the average of three independent experiments with standard deviation is shown. **P*<0.05, calculated using Student's *t*-test. Scale bars: 100 μm in A,D; 200 μm in G.

neurogenesis by measuring the outer edges of the βIII-tubulin domains in the central retinal sections confirmed the delay of neurogenesis in the Sox11-KO retina until E13 (Fig. 3C). Cone photoreceptors are one of earliest differentiated retinal cell subtypes. At E13, signals for the cone marker RXRγ were observed in the control retina. By contrast, the RXRγ signal in the Sox11-KO retina was weak, and the number of positive cells was about half that in the control retina (Fig. 3D). At E18, numbers of RXRγ-positive cells were comparable in Sox11-KO and control retinas (Fig. 3D). Similarly, HuC/D-positive amacrine cells and NF160-positive horizontal cells, which are absent at around E12 in Sox11-KO retinas, were present in similar numbers in Sox11-KO and control retinas at E18 (data not shown).

Sox11-KO retinas develop relatively normally at the postnatal stage

Because Sox11-KO mice die at birth (Sock et al., 2004), we examined postnatal retinal development by explant culture. E18 retinas were isolated and cultured as explants for 2 weeks. Frozen sections were then immunostained. HuC/D, calbindin 28k (horizontal cells), RXRγ, PNR, Chx10 (bipolar cells), GS and cyclin D3 (Müller glia) showed similar expression patterns in Sox11-KO and control retinas (supplementary material Fig. S3), suggesting that postnatal differentiation of Sox11-KO retinal cells was normal.

Proliferation of retinal cells was only slightly perturbed in Sox11-KO retinas

As Sox11-KO retinas showed microphthalmia, we next examined proliferation in Sox11-KO retinas by measuring BrdU incorporation and expression of phospho-histone H3, an M-phase marker (Fig. 4A). Although the diameters of Sox11-KO retinas were much smaller than those of control retinas, numbers of BrdU-positive cells (Fig. 4A,B) and phospho-histone H3-positive cells (Fig. 4A,C) were comparable at E11-E13. Numbers of phospho-histone H3-positive cells were also comparable at E14 and E18 (data not shown). We then examined BrdU and phospho-histone H3 double-positive cells at E12 after *in utero* BrdU labeling for 2 or 4 hours (Fig. 4D). Incorporation of BrdU was comparable in control and Sox11-KO retinas (Fig. 4D,E), and the number of BrdU- and phospho-histone H3-double-positive cells was also comparable (Fig. 4F). Taking these results together, we conclude that ablation of Sox11 delayed retinal cell differentiation without perturbing cell proliferation. We also investigated apoptotic cells

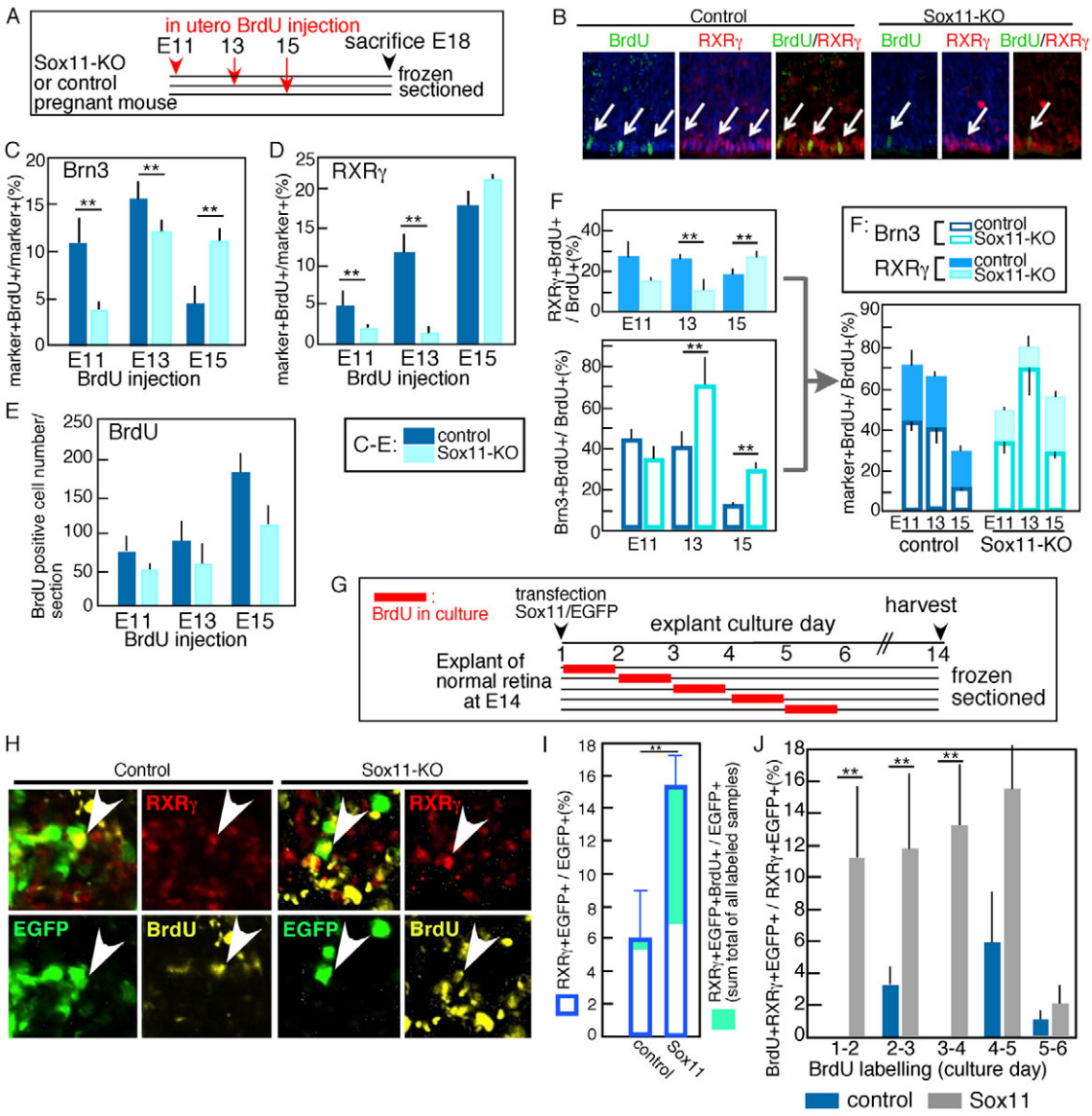

Fig. 5. Birth-date analysis of loss- and gain-of-function of Sox11. (**A-F**) For BrdU labeling to Sox11-KO embryo, pulse labeling of BrdU was conducted by injection of BrdU into pregnant females intraperitoneally at timing indicated in A, and embryos were sacrificed at E18. Eyes were frozen sectioned and double stained with anti-BrdU and markers (Brn3b or RXRγ). Marker and BrdU double-positive cells (C,D), BrdU-positive cells (E) and double-positive cells out of total BrdU-positive cells (F) are shown. (**G-J**) Birth-date analysis was carried out for gain-of-function Sox11 retina. At E14, retinas were isolated and transfected with Sox11-EGFP expression plasmid. Then retinas were cultured as explants, and BrdU was present every 24 hours, as indicated schematically in G. (H) Retinas were harvested after 2 weeks. The sections were triple immunostained with anti-EGFP, anti-BrdU and anti-RXRγ. (I) The percentage of RXRγ and EGFP double-positive cells in EGFP single-positive cells over a total of 6 days' labeling are shown. BrdU-positive and -negative populations in RXRγ/EGFP cells are shown in different colors. Percentage of RXRγ/EGFP and BrdU-positive cells in EGFP cells are calculated in each sample and a summary of the results is shown. (J) The percentage of triple-positive cells out of double-positive cells on each BrdU labeling day. $^{**}P<0.01$, Student's *t*-test. Data are mean+s.d.

by examining active caspase 3-positive cells (Fig. 4G,H). The number of apoptotic cells was higher in Sox11-KO retinas at all stages we examined (E12, E14 and E18), suggesting that Sox11 may play important roles in cell survival during retinal development. The microphthalmia observed in Sox11-KO retinas may be partly explained by increased apoptosis. No difference was seen in the number of apoptotic cells when Sox11 was overexpressed (Fig. 4I). We surmise that high expression of endogenous Sox11 in the early developmental period may explain why we did not observe fewer apoptotic cells when Sox11 was overexpressed.

Birth dates of cone and ganglion cell were affected by the level of Sox11

As modulating the level of Sox11 perturbs the differentiation of retinal cell subtypes, we examined whether birth date of these cells was affected by Sox11. We first examined whether the generation of cone and ganglion cells was affected in Sox11-KO retinas by pulse-labeling with BrdU at different embryonic stages. BrdU was administrated intraperitoneally to pregnant Sox11-KO mice at E11, E13 or E15, and embryos were harvested at E18 (Fig. 5A). BrdU- and marker-positive cells were examined by immunostaining of frozen eye sections (Fig. 5B). Ten percent of the ganglion cells

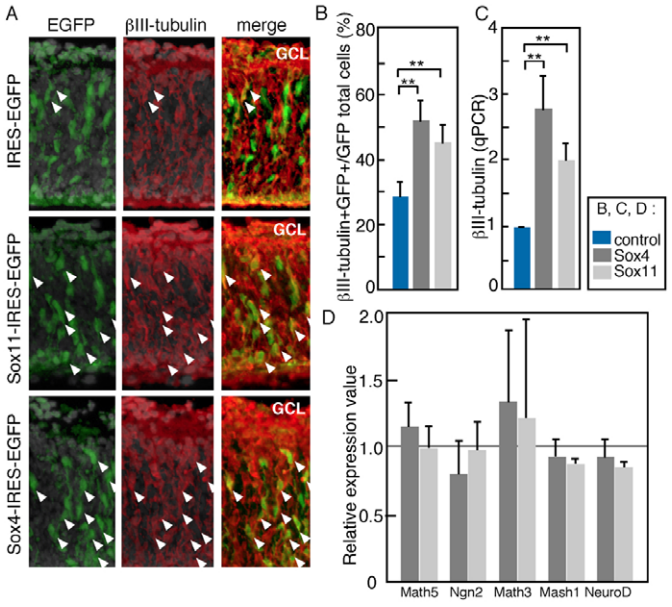

Fig. 6. Sox11 and Sox4 induced βIII-tubulin expression but not that of other genes. (**A**,**B**) Examination of molecules induced by Sox11 or Sox4. (A) Retrovirus encoding Sox11-, Sox4-IRES-EGFP or control EGFP was transduced into retinal explants at E16. On the third day, frozen sections were made and stained with antibody against-EGFP and -βIII-tubulin. (B) Percentage of EGFP and βIII-tubulin double-positive cells in total EGFP-positive cells. (**C**,**D**) Sox11-IRES-EGFP, Sox4-IRES-EGFP or control EGFP was electroporated to retinas at E16, and, after culture for 48 hours, EGFP-positive cells were collected by a cell sorter and qRT-PCR was carried out. Relative expression of βIII-tubulin (C) and indicated genes (D) in EGFP-positive cells are shown. Values relative to control plasmid transfected samples are shown $**P<0.01$ (Student's *t*-test). Data are mean+s.d.

(labeled with Brn3) were positive for BrdU in control retinas at E11. By contrast, fewer than 5% of these cells were positive for BrdU in the Sox11-KO retina (Fig. 5C). With labeling at E13, 15% of Brn3-positive cells were BrdU-positive in the control retina, and more than 10% of Brn3-positive cells were BrdU positive in Sox11-KO retinas (Fig. 5C). With labeling at E15, more than 10% of ganglion cells were BrdU positive in the Sox11-KO retina whereas fewer than 5% were positive in the control retina (Fig. 5C). In the case of cone cells, only a small proportion of cells were generated at E11 and E13 in the Sox11-KO retina, in contrast to the control retina (Fig. 5D). However, at E15, comparable numbers of cone cells were BrdU positive in control and Sox11-KO retinas. The number of BrdU-positive cells increased in a similar manner from E11 to E15 in both control and Sox11-KO retinas (Fig. 5E). However, when we calculated the population of ganglion and cone cells born in total BrdU-positive cells at each stage (Fig. 5F), it was apparent that the birth dates of cone and ganglion cells shifted to later stages in Sox11-KO retina, with the onset and end of cone birth both delayed in the Sox11-KO retina.

We next examined whether overexpression of Sox11 affected the birth date of cone cells. Plasmids encoding Sox11 and EGFP were introduced into retinal explants prepared from retinas at E14, and BrdU was applied for 24 hour at different timings in explant culture, as shown schematically in Fig. 5G. Explants were harvested after 2 weeks and incorporation of BrdU, and expression of cone markers and EGFP, were examined by triple immunostaining (Fig. 5H). We first confirmed that cones were produced in greater numbers when Sox11 was overexpressed, but the numbers were not as high as when Sox11 was introduced at E17 (Fig. 5I, blue empty bar; RXRγ+EGFP+/EGFP+). This may be explained by high endogenous expression of Sox11 and ongoing cone genesis in retina at E14, such that ectopic Sox11 expression might provide only a moderate boost. Triple staining with BrdU, RXRγ and EGFP showed that for all the BrdU-labeling periods examined, the number of labeled cone cells was higher than that of control (Fig. 5J), suggesting that Sox11 overexpression leads to cone genesis, even after the normal cone genesis. We surmise that the non-staining cone population may correspond to cones born during the initial 24 hours of culture. We first determined the RXRγ+EGFP+BrdU+/EGFP+ population in each labeled sample (five samples) and determined the sum of these values (Fig. 5I, green region). The value of the non-stained cell population (Fig. 5I, noncolored values), which represents RXRγ+EGFP+BrdU cells in the total EGFP+ cell population, was about 6% in both control and Sox11-overexpressing samples (Fig. 5I, noncolored values of blue bars). This suggests that at E14, cone genesis is comparable in control and Sox11-overexpressing samples. Cone genesis rapidly declines thereafter in controls, whereas it continues in Sox11-overexpressing samples. Therefore, with Sox11 overexpression, cone genesis began at the normal time but did not cease at the appropriate time, instead continuing through to a later stage, resulting in an excess number of cone cells.

Overexpression of Sox11 or Sox4 did not induce expression of genes, with the exception of βIII-tubulin

We next tested whether Sox11 and Sox4 induce Math5 (Atoh7 – Mouse Genome Informatics), Ngn2 (Neurog2 – Mouse Genome Informatics) and Math3 (Neurod4 – Mouse Genome Informatics) expression. As a positive control, we first examined βIII-tubulin, which is known to be a direct target of Sox11 and Sox4 (Bergsland et al., 2006; Dy et al., 2008). Sox11-IRES-EGFP or Sox4-IRES-EGFP was introduced into retinas at E16, and after 4 days of culture, βIII-tubulin and EGFP were immunostained (Fig. 6A). Sox11 and Sox4 each increased the number of βIII-tubulin-positive cells (Fig. 6A,B). After 2 days of culture, βIII-tubulin expression was examined by qPCR (Fig. 6C). As expected, βIII-tubulin mRNA expression was induced both by Sox11 and Sox4. Using the same samples, we tested whether Sox11 and Sox4 promote neurogenesis by examining the expression of genes related to neural fate. Expression of the proneural bHLH genes – Math5, Ngn2, Mash1 (Ascl1 – Mouse Genome Informatics), NeuroD (Neurod1 – Mouse Genome Informatics) and Math3, all of which are expressed by retinal precursor cells as intrinsic regulators of retinal cell fate decision (Akagi et al., 2004; Cepko, 1999) – was not induced by Sox11 or Sox4 overexpression (Fig. 6D).

Global transcriptional effects of loss of Sox11 function

To delineate the molecular mechanisms underlying the retinal action of Sox11, we performed microarray analysis of E18 retinas from wild-type and Sox11-KO mice. Genes with a greater than halving of expression are listed in supplementary material Table S5A; those with a greater than twofold increase in expression are given in supplementary material Table S5B. Expression of S-antigen (arrestin; Mm.1276), PNR (Mm.103641), and Nrl (Mm.20422) decreased by more than half; in Sox11-KO retinas, suggesting that differentiation of rod photoreceptors was delayed in Sox11-KO retinas. Expression

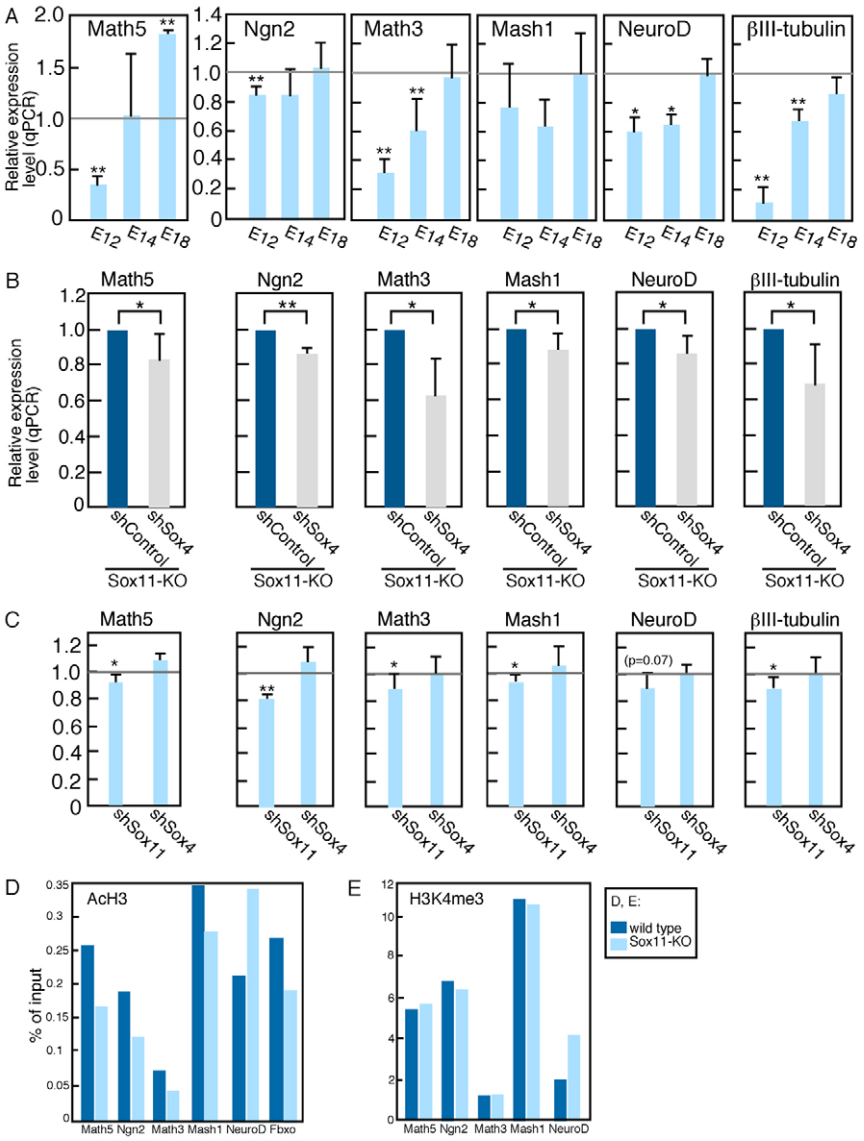

Fig. 7. Knockdown of Sox4 in Sox11-KO retina suppressed recovery of gene expression. (**A**) Expression of various genes in developing Sox11-KO retina. Expression of indicated genes of Sox11-KO or control littermate was examined by qPCR. Expression levels relative to values in control retina at matched ages are shown. (**B**) Expression of βIII tubulin and proneural genes in Sox11-KO retina transfected with shRNA for Sox4. (**C**) Expression of βIII tubulin and proneural genes in the normal retina expressing sh-Sox11 or sh-Sox4. Plasmids encoding sh-Sox11 or -Sox4-IRES-EGFP were transfected in to normal retina at E14, and the retinas were cultured for 3 days. The expression of indicated genes was examined by qPCR. (**D**,**E**) ChIP analysis for AcH3 (D) and HeK4me3 (E) was carried out using retinas at E16 of Sox11-KO or control littermate. $^{**}P<0.01$, $^{*}P<0.05$ (Student's *t*-test). Data are mean+s.d.

of Math5 (Mm.228661), Dlx1 (Mm.475101) and Dlx2 (Mm.3896), important genes in the differentiation of ganglion cells (de Melo et al., 2005; Wang et al., 2001; Yang et al., 2003), increased more than twofold in the Sox11-KO retina, showing that a delay in ganglion cell differentiation had occurred by the onset of Sox4 expression, and that related genes were expressed at E18, a stage by which they had already been shut off in normal retinas (data not shown).

Knockdown of Sox4 in Sox11-KO retina prevented the recovery of gene expression

The onset of βIII-tubulin protein expression was delayed in the Sox11-KO retina (Fig. 3A,C). The expression of βIII-tubulin mRNA in the Sox11-KO retina was much lower at E12 and E14. At E18, the level was comparable with that in controls (Fig. 6A), suggesting that βIII-tubulin expression is regulated by Sox11 in the early developmental stage. The proneural bHLH genes Ngn2, Math3, Mash1 and NeuroD were also expressed at lower levels in the Sox11-KO retina (Fig. 7A). At later stages, the expression of βIII-tubulin and proneural genes was restored in the Sox11-KO retina (Fig. 7A). We surmised that this recovery may have been caused by the onset of Sox4 expression. To test this possibility, we introduced a Sox4-specific shRNA (sh-Sox4) into E16 Sox11-KO retinas by electroporation and culturing the retinas for 2 days as explants. The expression of βIII-tubulin and proneural genes was reduced in sh-Sox4-treated retinas (Fig. 7B), supporting the notion that Sox4 participates in the restoration of βIII-tubulin expression. As retina from Sox11-KO mice showed anterior segment dysgenesis (Wurm et al., 2008), it is possible that the retinal defects described in our work are secondary. We therefore expressed sh-Sox11 in normal retina, and examined its effects on gene expression by qPCR (Fig. 7C). We first confirmed suppressive effects of sh-So11 in retina (supplementary material Fig. S4A). We found that expression of proneural genes was reduced in the presence of sh-Sox11, but found no effect for sh-Sox4, probably because Sox4 is not significantly expressed at around the E14 stage in retina. In accordance with this observation, when we extended culture of sh-Sox11-expressing retina to 7 days, repression of the genes was not observed (data not shown) probably owing to expression of Sox4 in later stage retinal development.

Based on our finding that Sox11 expression did not induce significant gene expression, we speculated that Sox11 may help to establish competency to allow differentiation of neurons. To

examine this hypothesis, we next assessed the histone modification status in promoters of selected genes. We performed chromatin immunoprecipitation (ChIP) analysis of acetyl histone H3 (AcH3) and histone H3K4 tri-methylation (H3K4me3), both of which positively regulate transcription, using E16 retinas from Sox11-KO mice and littermates. AcH3 levels for Math5, Ngn2, Math3 and Mash1 promoters were lower in Sox11-KO (Fig. 7D). The values for H3K4me3 were comparable in Sox11-KO and control retinas (Fig. 7E), except for the NeuroD promoter, which showed a higher value in Sox11-KO retinas. We further analyzed whether the epigenetic status of genes identified in the DNA microarray analysis was modified in Sox11-KO retina. We examined Nrl, Nudt21, Fbxo2, Nr2e3, Bcan, Adi1, Sag, Tcfap2 and Camk2b. However, significant, reproducible differences were not observed, except for Fbxo and Adi1. In the Sox11-KO retina, acetylated histone AcH3 levels in the Fbxo locus were reduced (Fig. 7D), whereas Adi1 H3K4me3 levels increased (Fig. 7E). Finally, we examined whether overexpression of Sox11 or Sox4 affects epigenetic modification of these genes. Using EGFP to monitor transformation, we overexpressed Sox11 or Sox4 in E15 retinas, and after 2 days of culture, EGFP-positive cells were purified using a cell sorter, and ChIP analysis was conducted for AcH3 and H3K4me3 modifications in the Ngn2, Math5, Math3, NeuroD and Mash1 loci. We observed no significant changes in AcH3 and H3K4me3 levels at the Ngn2 and Math3 loci. For NeuroD and Mash1, the levels of both AcH3 and H3K4me3 were downregulated by Sox11 (supplementary material Fig. S4B). With Sox4, AcH3 was downregulated in the NeuroD locus. As the level of AcH3 for NeuroD was increased in Sox11-KO (Fig. 7D), we inferred a role for Sox11 in the AcH3 modification of NeuroD and continued to work to uncover the mechanisms.

DISCUSSION

We found that Sox11 and Sox4 have unique patterns of expression during retinal development, and that perturbation of the expression pattern of either Sox11 or Sox4 disrupts differentiation of subsets of retinal cells without affecting the proliferative activity of retinal progenitor cells. Therefore, the altered differentiation is probably caused by the direct effects of Sox11 and Sox4 on retinal cell fate decision, rather than secondary effects resulting from perturbation of the timing of cell cycle exit. In adult mouse hippocampus, Sox4/Sox11 ablation decreased the generation of cells expressing neuron-specific proteins, without significant alterations in proliferation (Mu et al., 2012). However, evidence showing involvement of Sox11 in mantle cell lymphoma had been accumulated (Sander, 2011), and more recently, involvement of Sox4 in lung cancers was reported (Castillo et al., 2012), thus suggesting that Sox11 and Sox4 differently involve proliferation in CNS and other tissues.

Sox11 and Sox4 have remarkable identity in the HMG box DNA-binding and the C-terminal transactivation domains, and their conserved molecular properties have been demonstrated previously (Dy et al., 2008). Our results demonstrate that Sox11 and Sox4 had nearly identical effects on retinal progenitor cell behavior when they were overexpressed. Therefore, phenotypic recovery of the Sox11-KO retina at or after birth may be explained by the compensatory effects of Sox4, which starts to be expressed at around E18.

As Sox11 is known to be a transcription activator, we tried to identify targets of Sox11 in the retina. βIII-tubulin is the only one whose expression was shown to be induced by ectopic expression of Sox11 or Sox4 in the retina. Initiation of βIII-tubulin and Ngn2 expression coincide in the mouse retina, and no differentiating retinal neurons are present prior to the onset of Ngn2 and βIII-tubulin expression (Hufnagel et al., 2010). Therefore, βIII-tubulin is a marker for the beginning of neurogenesis in the retina; however, as far as we know, no report has suggested that βIII tubulin acts as a master regulator to initiate neurogenesis. Therefore, the βIII-tubulin yet not give retinal progenitors the cue to start differentiating. Recently, Fezf2 and BDNF were found as targets of Sox11 in cortex and dorsal root ganglia, respectively (Salerno et al., 2012; Shim et al., 2012). Our microarray data of Sox11-KO retina showed rather elevated expression of Fezf2 and BDNF (data not shown), suggesting cell type-specific activation of genes by Sox11.

Comparative microarray analysis of Sox11-KO and wild-type retinas revealed global transcriptional effects as a consequence of the loss of Sox11 function. However, overexpression experiments identified only βIII-tubulin as a possible target gene of Sox11 and Sox4. Therefore, we surmise that Sox11 may help cells to acquire competence for stage-specific differentiation, rather than directly activating the transcription of genes that are crucial to retinal differentiation. Our finding that the histone acetylation of several genes was altered in the Sox11-KO retina supports this hypothesis. Based on these results alone, we cannot attribute the gain-of-function phenotype to changes in the epigenetic statuses of the genes. We surmise that a set of genes is epigenetically regulated by Sox11, but why they are selectively regulated remains to be clarified. A recent paper (Bergsland et al., 2011) showed sequential roles of Sox3 and Sox11 in neural lineage development, and a model that bivalent histone modifications of specific genes had been established by sequential Sox protein bindings in target genes. We are currently attempting to determine the molecular mechanisms by which Sox11 and Sox4 regulate epigenetic status in the retina.

Strong suppression of differentiation of Müller glia by Sox11 and Sox4 was observed. Notch signaling is known to promote differentiation in the Müller glia lineage in the retina (Furukawa et al., 1997; Hojo et al., 2000), but our results suggest that Sox11 and Sox4 may not directly suppress Notch. Instead, they may suppress the expression of genes that play roles important in glia differentiation. Recently, the results of comprehensive analysis of expression patterns in purified Müller glia lineage cells from P0 to P21 were reported (Nelson et al., 2011). We chose several genes from the microarray result and examined their expression in Sox11- or Sox4-overexpressing retinal cells by qPCR and in the Sox11-KO retina using microarrays. We identified several genes that are up- or downregulated in the Sox11-KO retina; but, in the Sox-overexpressing cells, only Bmpr1a expression was found to be enhanced (data not shown). The roles of BMP signals in mammalian Müller glia differentiation have been reported in chick (Huillard et al., 2005), and we are currently clarifying the participation of BMP signaling in Müller glia differentiation in conjunction with Sox11.

The forced activation of Notch signaling has been shown to suppress cone photoreceptor fate specification (Jadhav et al., 2006; Riesenberg et al., 2009; Yaron et al., 2006). However, as discussed above, direct suppression of Notch signaling by Sox11 or Sox4 is unlikely. We examined whether Sox11 and Sox4 induce the expression of cone- and photoreceptor-lineage related genes such as *Nrl*, *Otx2*, and TRβ2 (Thrb2 – Mouse Genome Informatics), but no induction in the retina was observed at E17 when Sox11 or Sox4 was overexpressed (data not shown). Therefore, analysis of epigenetic regulation seems promising as an approach to learning about the mechanisms of cone induction, and further studies are currently under way.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References

Akagi, T., Inoue, T., Miyoshi, G., Bessho, Y., Takahashi, M., Lee, J. E., Guillemot, F. and Kageyama, R. (2004). Requirement of multiple basic helix-loop-helix genes for retinal neuronal subtype specification. *J. Biol. Chem.* **279**, 28492-28498.
Bergsland, M., Werme, M., Malewicz, M., Perlmann, T. and Muhr, J. (2006). The establishment of neuronal properties is controlled by Sox4 and Sox11. *Genes Dev.* **20**, 3475-3486.
Bergsland, M., Ramsköld, D., Zaouter, C., Klum, S., Sandberg, R. and Muhr, J. (2011). Sequentially acting Sox transcription factors in neural lineage development. *Genes Dev.* **25**, 2453-2464.
Bowles, J., Schepers, G. and Koopman, P. (2000). Phylogeny of the SOX family of developmental transcription factors based on sequence and structural indicators. *Dev. Biol.* **227**, 239-255.
Castillo, S. D., Matheu, A., Mariani, N., Carretero, J., Lopez-Rios, F., Lovell-Badge, R. and Sanchez-Cespedes, M. (2012). Novel transcriptional targets of the SRY-HMG box transcription factor SOX4 link its expression to the development of small cell lung cancer. *Cancer Res.* **72**, 176-186.
Cepko, C. L. (1999). The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. *Curr. Opin. Neurobiol.* **9**, 37-46.
de Melo, J., Du, G., Fonseca, M., Gillespie, L. A., Turk, W. J., Rubenstein, J. L. and Eisenstat, D. D. (2005). Dlx1 and Dlx2 function is necessary for terminal differentiation and survival of late-born retinal ganglion cells in the developing mouse retina. *Development* **132**, 311-322.
Dy, P., Penzo-Méndez, A., Wang, H., Pedraza, C. E., Macklin, W. B. and Lefebvre, V. (2008). The three SoxC proteins – Sox4, Sox11 and Sox12 – exhibit overlapping expression patterns and molecular properties. *Nucleic Acids Res.* **36**, 3101-3117.
Furukawa, T., Kozak, C. A. and Cepko, C. L. (1997). rax, a novel paired-type homeobox gene, shows expression in the anterior neural fold and developing retina. *Proc. Natl. Acad. Sci. USA* **94**, 3088-3093.
Harris, W. A. (1997). Cellular diversification in the vertebrate retina. *Curr. Opin. Genet. Dev.* **7**, 651-658.
Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F. and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. *Development* **127**, 2515-2522.
Hoser, M., Potzner, M. R., Koch, J. M., Bösl, M. R., Wegner, M. and Sock, E. (2008). Sox12 deletion in the mouse reveals nonreciprocal redundancy with the related Sox4 and Sox11 transcription factors. *Mol. Cell. Biol.* **28**, 4675-4687.
Hufnagel, R. B., Le, T. T., Riesenberg, A. L. and Brown, N. L. (2010). Neurog2 controls the leading edge of neurogenesis in the mammalian retina. *Dev. Biol.* **340**, 490-503.
Huillard, E., Laugier, D. and Marx, M. (2005). Defects in chicken neuroretina misexpressing the BMP antagonist Drm/Gremlin. *Dev. Biol.* **283**, 335-344.
Iida, A., Shinoe, T., Baba, Y., Mano, H. and Watanabe, S. (2011). Dicer plays essential roles for retinal development by regulation of survival and differentiation. *Invest. Ophthalmol. Vis. Sci.* **52**, 3008-3017.
Jadhav, A. P., Mason, H. A. and Cepko, C. L. (2006). Notch 1 inhibits photoreceptor production in the developing mammalian retina. *Development* **133**, 913-923.
Koso, H., Ouchi, Y., Tabata, Y., Aoki, Y., Satoh, S., Arai, K. and Watanabe, S. (2006). SSEA-1 marks regionally restricted immature subpopulations of embryonic retinal progenitor cells that are regulated by the Wnt signaling pathway. *Dev. Biol.* **292**, 265-276.
Koso, H., Satoh, S. and Watanabe, S. (2007). c-kit marks late retinal progenitor cells and regulates their differentiation in developing mouse retina. *Dev. Biol.* **301**, 141-154.
Koso, H., Iida, A., Tabata, Y., Baba, Y., Satoh, S. S., Taketo, M. M. and Watanabe, S. (2008). CD138/syndecan-1 and SSEA-1 mark distinct populations of developing ciliary epithelium that are regulated differentially by Wnt signal. *Stem Cells* **26**, 3162-3171.
Marquardt, T. and Gruss, P. (2002). Generating neuronal diversity in the retina: one for nearly all. *Trends Neurosci.* **25**, 32-38.
Minoguchi, S., Taniguchi, Y., Kato, H., Okazaki, T., Strobl, L. J., Zimber-Strobl, U., Bornkamm, G. W. and Honjo, T. (1997). RBP-L, a transcriotion factor related to RBP-Jkappa. *Mol. Cell Biol.* **17**, 2679-2687.
Mu, L., Berti, L., Masserdotti, G., Covic, M., Michaelidis, T. M., Doberauer, K., Merz, K., Rehfeld, F., Haslinger, A., Wegner, M. et al. (2012). SoxC transcription factors are required for neuronal differentiation in adult hippocampal neurogenesis. *J. Neurosci.* **32**, 3067-3080.
Nelson, B. R., Ueki, Y., Reardon, S., Karl, M. O., Georgi, S., Hartman, B. H., Lamba, D. A. and Reh, T. A. (2011). Genome-wide analysis of Müller glial differentiation reveals a requirement for Notch signaling in postmitotic cells to maintain the glial fate. *PLoS ONE* **6**, e22817.
Riesenberg, A. N., Liu, Z., Kopan, R. and Brown, N. L. (2009). Rbpj cell autonomous regulation of retinal ganglion cell and cone photoreceptor fates in the mouse retina. *J. Neurosci.* **29**, 12865-12877.
Salerno, K. M., Jing, X., Diges, C. M., Cornuet, P. K., Glorioso, J. C. and Albers, K. M. (2012). Sox11 modulates brain-derived neurotrophic factor expression in an exon promoter-specific manner. *J. Neurosci. Res.* **90**, 1011-1019.
Sander, B. (2011). Mantle cell lymphoma: recent insights into pathogenesis, clinical variability, and new diagnostic markers. *Semin. Diagn. Pathol.* **28**, 245-255.
Schepers, G. E., Teasdale, R. D. and Koopman, P. (2002). Twenty pairs of sox: extent, homology, and nomenclature of the mouse and human sox transcription factor gene families. *Dev. Cell* **3**, 167-170.
Shim, S., Kwan, K. Y., Li, M., Lefebvre, V. and Sestan, N. (2012). Cis-regulatory control of corticospinal system development and evolution. *Nature* **486**, 74-79.
Sock, E., Rettig, S. D., Enderich, J., Bösl, M. R., Tamm, E. R. and Wegner, M. (2004). Gene targeting reveals a widespread role for the high-mobility-group transcription factor Sox11 in tissue remodeling. *Mol. Cell. Biol.* **24**, 6635-6644.
Tabata, Y., Ouchi, Y., Kamiya, H., Manabe, T., Arai, K. and Watanabe, S. (2004). Retinal fate specification of mouse embryonic stem cells by ectopic expression of Rx/rax, a homeobox gene. *Mol. Cell. Biol.* **24**, 4513-4521.
Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. *Genes Dev.* **15**, 24-29.
Wurm, A., Sock, E., Fuchshofer, R., Wegner, M. and Tamm, E. R. (2008). Anterior segment dysgenesis in the eyes of mice deficient for the high-mobility-group transcription factor Sox11. *Exp. Eye Res.* **86**, 895-907.
Yang, Z., Ding, K., Pan, L., Deng, M. and Gan, L. (2003). Math5 determines the competence state of retinal ganglion cell progenitors. *Dev. Biol.* **264**, 240-254.
Yaron, O., Farhy, C., Marquardt, T., Applebury, M. and Ashery-Padan, R. (2006). Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. *Development* **133**, 1367-1378.

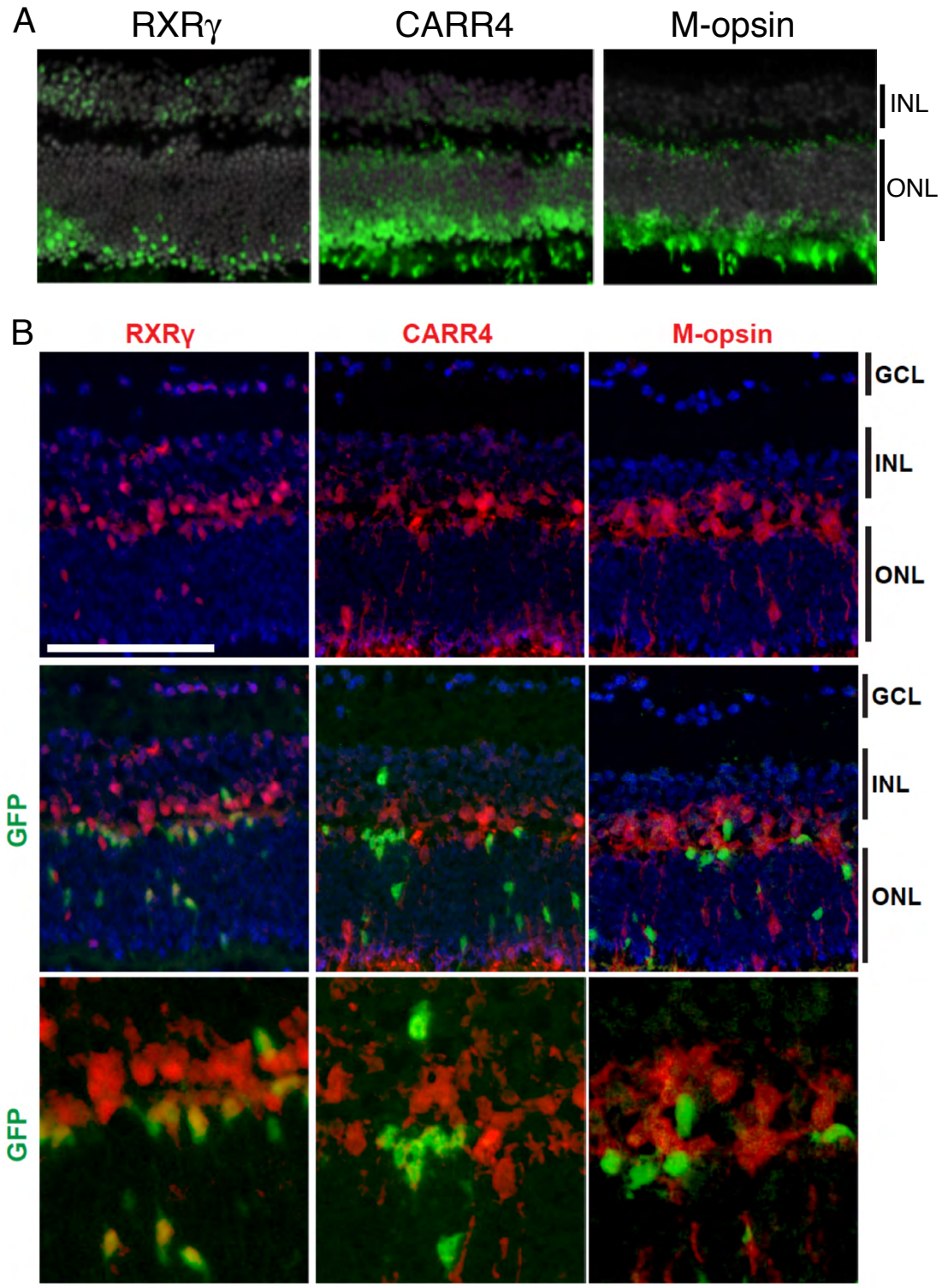

Fig. S1. Immunostaining of Sox11/Sox4 overexpressed retinal sections by antibodies anti-M-opsin and CARR4. (**A**) Frozen sectioned adult retina was immunostained with indicated antibodies. Nucleus was stained with DAPI and expressed in gray. (**B**) Mouse retina at E17 was transfected with Sox11-EGFP, cultured as an explant for 2 weeks and then frozen sectioned. Immunostaining using anti-EGFP, RXRγ, CARR4 and M-opsin was carried out. Nucleus was stained with DAPI and expressed in blue. CARR4 and M-opsin antibodies recognized cells in the border between INL and ONL, confirming that these cells are cone. However, although RXRγ merged with EGFP-positive cells, CARR4 and M-opsin antibodies did not merge with the cells. These results suggest that the cells are of the cone lineage but did not achieve final maturation.

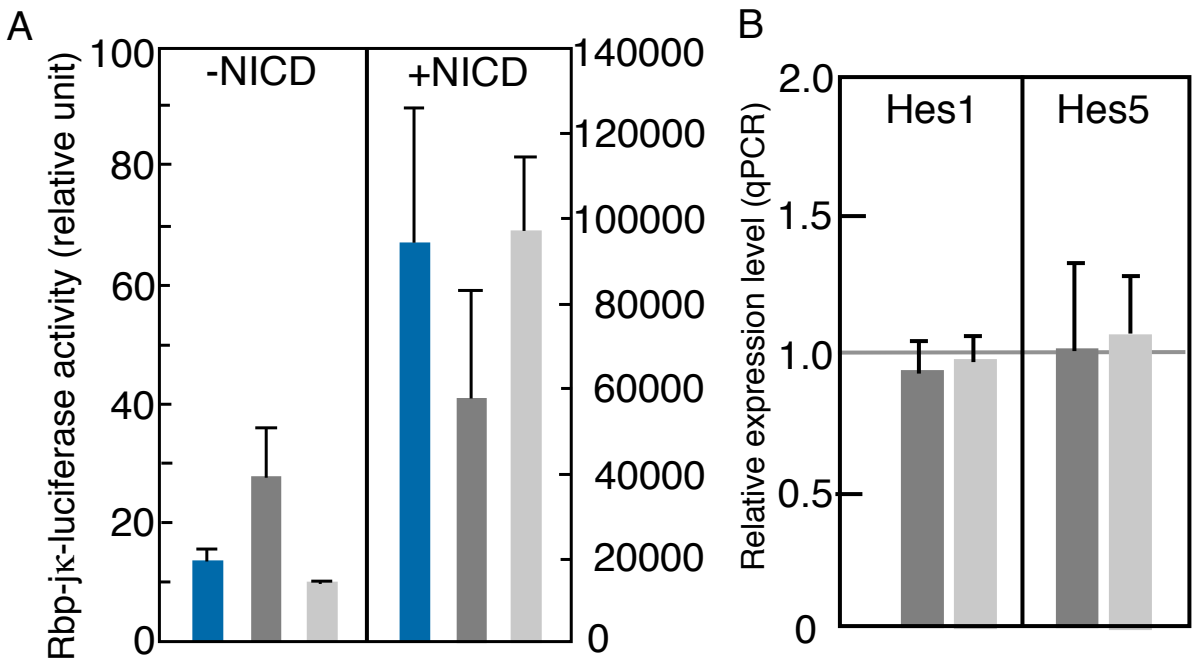

Fig. S2. Effects of Sox11 and Sox4 for Notch signaling. (**A**) Effects of Sox11 and Sox4 on Notch signaling were examined by luciferase analysis. Y79 cells were transfected with Rbp-jk-luciferase plasmid with pCAG-Sox11, pCAG-Sox4 or control pCAG, and pEF-NICD or control pEF plasmids. After 48 hours, luciferase activities were measured. (**B**) Retrovirus encoding Sox11-, Sox4-IRES-EGFP or control EGFP was transduced into retinal explants at E16 prepared from ICR mice. On the fourth day, the expression of Hes1 and Hes5 was examined by qPCR. Expression levels relative to values of control plasmid transfected samples are shown.

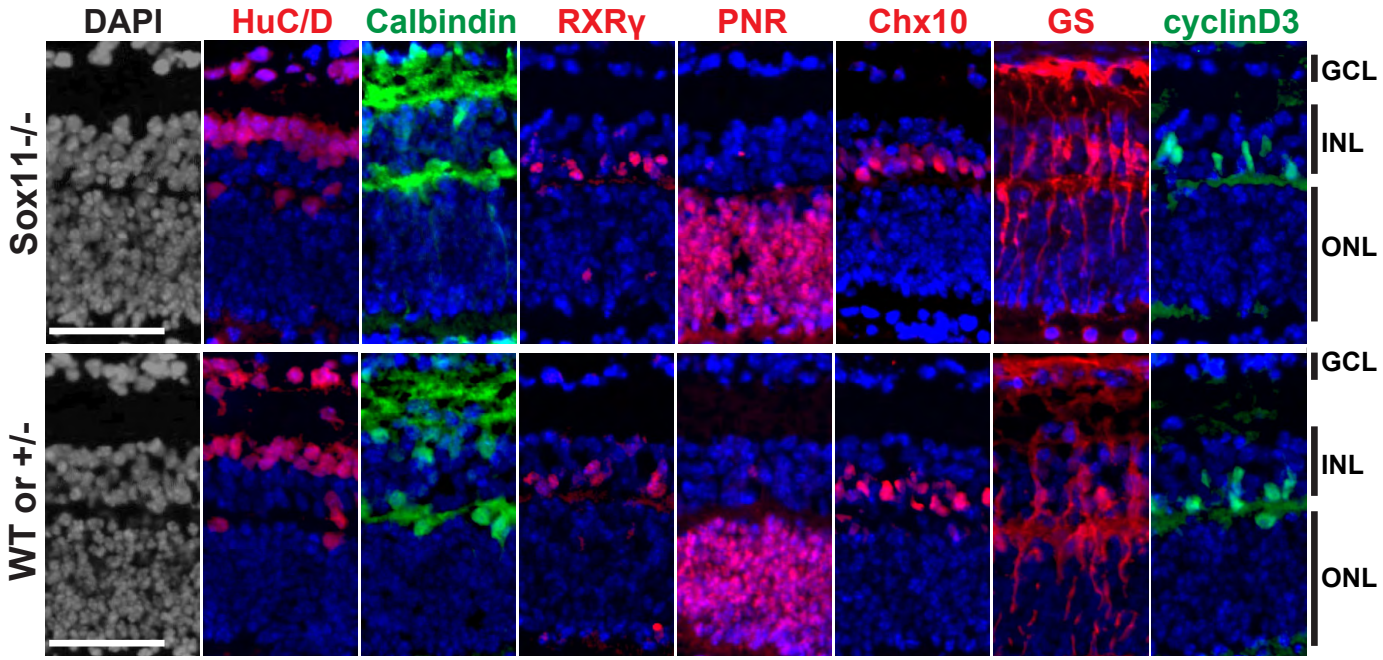

Fig. S3. The late phase of retinal differentiation was correct in Sox11-KO retinas. Retinas of Sox11-KO or control mice were isolated at E18 and cultured as explant cultures for 2 weeks. Retinas were harvested and frozen sectioned. The indicated markers were immunostained. Nuclei were visualized with DAPI. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bars: 50 μm.

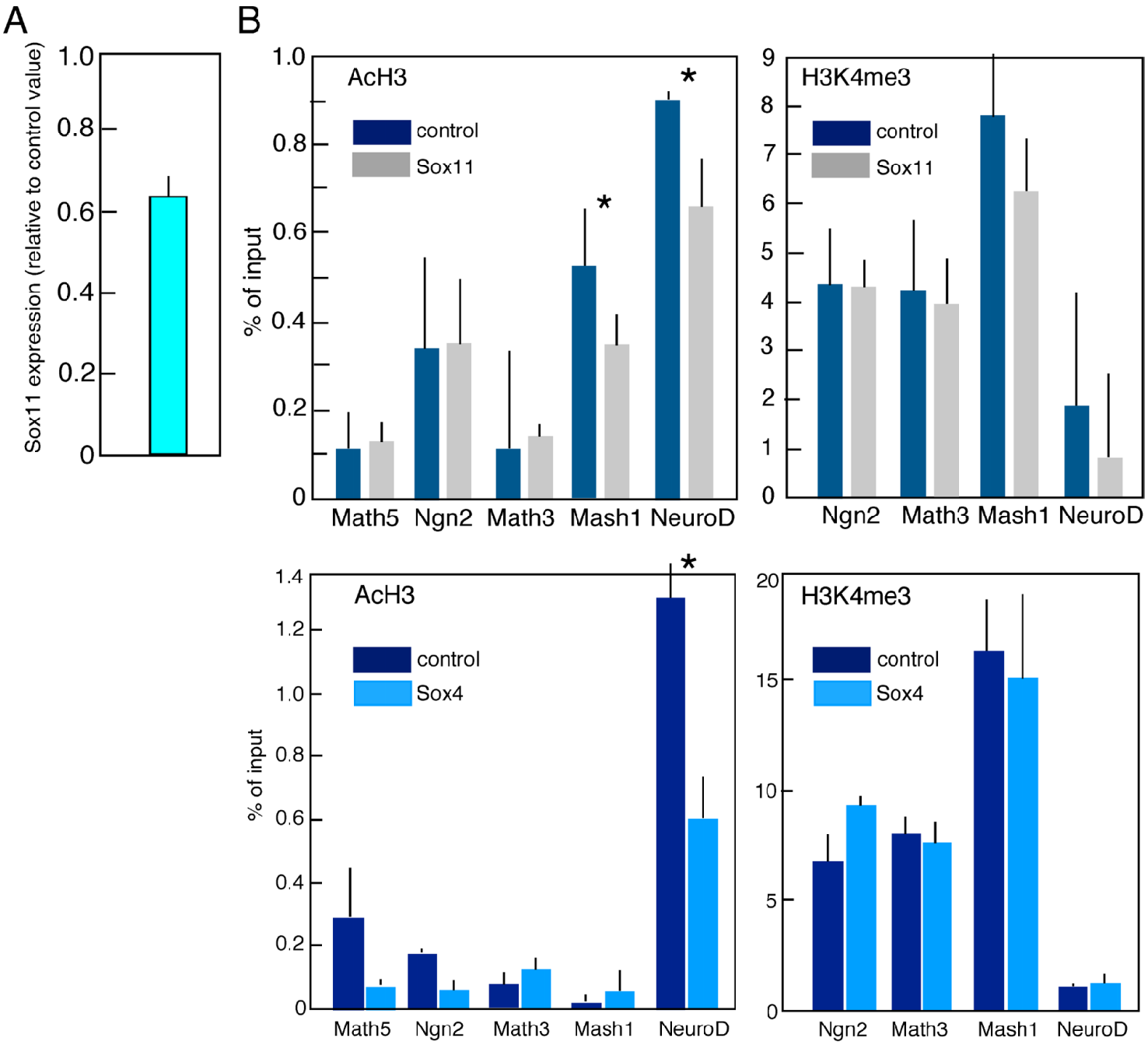

Fig. S4. Effects of Sox11 or Sox4 overexpression for histone modification of genes. (**A**) Effects of sh-Sox11 on expression levels of Sox11 in retina. Electroporation of sh-Sox11 was carried out using retinas at E15, and the retina was cultured for 3 days. Expression levels of Sox11 were examined by qPCR. Results are average of four independent samples ±s.d. (**B**) Effects of Sox11 or Sox4 overexpression on the histone modification of genes. Expression plasmid of Sox11-EGFP or Sox1-EGFP was introduced into retina (~20 retinas for one sample) at E15 by electroporation, and retinas were cultured for 2 days as explants. Retinas were then treated with trypsin to dissociate into single cells, and EGFP-positive cells were purified using a cell sorter and ~1×10^6 purified cells were served for each ChIP sample. Modification of AcH3 and H3K4me3 levels in Math5, Ngn2, Math3, Maxh1 and NeuroD enhancer region was analyzed by ChIP analysis. We repeated the same design of experiments at least three times, but some of results were not stable. By repeated experiments, reproducible results are shown in this figure. *P<0.05.

Table S1. Antibodies

Primary antibodies

Mouse monoclonal antibodies

βIII tubulin (Covance)
PNR (ppmx)
glutamine synthetase (GS, Chemicon)
HuC/D (Molecular Probes)
Ki67 (BD Bioscience)
BrdU (Roche)
kip1/p27(BD Bioscience)
NF160 (Sigma Aldrich)
Cyclin D3 (Santa Cruz)
β-galactocidase (DSHB)
Islet-a (DSHB)

Rabbit polyclonal antibodies

GFP (Clontech)
β-galactosidase (Cappel)
Sox4 (Abcam)
Calbindin D28K (Millipore)
Phospho histone H3 (Upstate)
RXRγ (Santa Cruz Biotechnology)
Active Caspase 3 (Promega)
Cone Arrestin 4 (Dr. Cheryl Craft, USC)
M-opsin (Dr. Cheryl Craft, USC)
Histone H3 (AcH3) antibody (Millipore)
Histone H3 tri-methyl Lys4 (H3K4me3) antibody (Activemotif)
rabbit control IgG (Santa Cruz)

Rat monoclonal antibody

GFP (Nakarai)

Sheep polyclonal antibody

Chx10 (Exalpha)

Goat polyclonal antibody

Brn3b (Santa Cruz Biotechnology)

Chicken polyclonal antibody

GFP (abcam)

Secondary antibodies

Alexa Fluor 488 goat anti-chicken IgG (H+L) (Invitrogen Molecular Probes)
Alexa Fluor 680 goat anti-mouse IgG (H+L) (Invitrogen Molecular Probes)
Alexa Fluor 568 goat anti-rabbit IgG (H+L) (Invitrogen Molecular Probes)

Table S2. Primers

RT-PCR

G3PDH: 5’- tgaccacagtccatgccatc -3’ and 5’- cataccaggaaatgagcttgac -3’
Sox11: ’- tgaaactcggtgcccaacag -3’ and 5’- attcgcggcttaaaggtccc -3’
Sox4: 5’- gagcttaggggagcattggc -3’ and 5’- gctgcaaggacaaggggaaa -3’
βIII tubulin: 5’-gctgtccgcctgcctttt -3’ and 5’- gacctcccagaacttggcc -3’
Math5: 5’- caggacaagaagctg -3’ and 5’- gggtctacctggagcc -3’
Hes1: 5’- atccgcagcaaactgcaaga-3’ and 5’- ggacactgtgtgggacccta -3’
Hes5: 5’- cttctgcgaagttcctggtc -3’ and 5’- atgtggaccttgaggtgagg-3’
Math3: 5’- attcagggctcgaagagtca -3’ and 5’- gttccttgccagtcgaagag-3’
Mash1: 5’- aacaaaccagacagccaacc -3’ and 5’- aggaacccatctgtgattcg-3’
NeuroD: 5’-caaagccacggatcaatctt -3’ and 5’- cccgggaatagtgaaactga-3’
Ngn2: 5’- tcgccagggactgtatct-3’ and 5’- ctgtgaagtggagtccg-3’

ChIP analysis

Primers for ChIP were designed around transcription start site
Math5: 5’-atccgcagcaaactgcaaga-3’, 5’-ggacactgtgtgggacccta-3’
Ngn2: 5’-gattgttttcttggtggtatataaggg-3’, 5’-gactccaaggcactccagttaaa-3’
Math3: 5’-aggaggtgtgtctttaggct-3’, 5’-cagctcctgtgatctgactg-3’
Sox11: 5’-tggggcccgtggttaataac-3’, 5’-tgagggctctctctcaactcg-3’
Sox4: 5’-cttctcattgcacgcggaga-3’, 5’-cagccgctgtaactaacgct-3’
Mash1: 5’-cagcagtctctcacttctgg-3’, 5'-tgagcctgcttctgggatta-3’
NeuroD: 5’-acagatgggccactttcttc-3’, 5'-atatggtcttcccggtccag-3’
Fbxo: 5’-ggaggaggaaagctccgata-3’, 5'-aggaggacatatggagtcgg-3’
Adi1: 5’-gcctttcctcctcctgaatt-3’, 5'-ttccactaactggctggaag-3’

Table S3. Comparison of expression level of members of SoxC group in different subsets of retinal progenitor cells

Gene	**Probe No**	**Unigene No.**	**SSEA1+(E14)**	**c-kit-(P1)**	**ckit+(P1)**
Sox11	1426790_a_at	Mm.41702	715.0	309.2	229.5
Sox4	1419155_a_at	Mm.240627	307.3	1471	1061.8
Sox12	1456882_at	Mm.28424	10.1	6.8	5.0

Table S4. Expression levels of members of SoxC group in retinal explant cultures

Gene	**Probe No**	**Unigene No.**	**E15**	**5 days cultured**
Sox11	1426790_a_at	Mm.41702	486.1	246.7
Sox4	1419155_a_at	Mm.240627	1082.7	1607.3
Sox12	1456882_at	Mm.28424	2.9	8.0

Table S5. Genes downregulated and upregulated in Sox11 knockout retina compared with littermate wild-type retina at E16

Genes are listed from smallest (Table 5A) and largest (Table 5B) fold change; the top 30 genes are shown.

A

Probe-set	Accession No.	Unigene	Gene	WT signal	KO signal	Fold change
1450813_a_at	NM_021467	Mm.44379	troponin I, skeletal, slow 1	33.4	2	0.06
1423232_at	X63190	Mm.5025	ets variant gene 4 (E1A enhancer binding protein, E1AF)	22.3	2.1	0.08
1431225_at	BB656631	Mm.466344	SRY-box containing gene 11	3205.7	252.4	0.08
1438648_x_at	AV069898	Mm.46387	gastrokine 3	29	1.8	0.11
1455869_at	BG862223	Mm.458283	calcium/calmodulin- dependent protein kinase II, beta	95.4	8	0.13
1419025_at	NM_009118	Mm.1276	retinal S-antigen	246.7	42.8	0.16
1438758_at	AU046270	Mm.291504	acireductone dioxygenase 1	129.4	20.1	0.16
1435197_at	BE993443	Mm.477577	POU domain, class 3, transcription factor 3	31.1	5.4	0.18
1451255_at	BC004672	Mm.4067	lipolysis stimulated lipoprotein receptor	33.8	12	0.19
1439143_at	BB312992	Mm.285300	RIKEN cDNA A930018M24 gene	28	3.7	0.19
1437121_at	BB168293	Mm.284447	DENN/MADD domain containing 1C	40.1	9	0.23
1449578_at	AW536705	Mm.479197	suppressor of Ty 16 homolog (S. cerevisiae)	157.7	41.5	0.27
1435205_at	C88013	Mm.331881	transcription factor AP-2, epsilon	61.4	14.6	0.27
1418726_a_at	NM_011619	Mm.247470	troponin T2, cardiac	47.5	15.6	0.33
1419740_at	NM_008806	Mm.1372	phosphodiesterase 6B, cGMP, rod receptor, beta	36.5	10.8	0.38
1423631_at	BB540797	Mm.103641	nuclear receptor subfamily 2, group E, member 3	179.3	76.3	0.38
1434369_a_at	AV016515	Mm.178	crystallin, alpha B	183.1	72.6	0.38
1429024_at	AK003783	Mm.479259	RNA binding motif protein 20	96.7	50.8	0.38
1425900_at	BC016235	Mm.213213	hexokinase domain containing 1	277.4	149.7	0.41
1427536_at	AI615965	Mm.442631	predicted gene 10330 /// zinc finger protein 125	49.8	12.9	0.41
1441899_x_at	BB335613	Mm.4598	brevican	57.9	13.9	0.41
1425892_a_at	D50055	Mm.16347	prepronociceptin	58.9	28.7	0.44
1431581_at	AK013705	---	RIKEN cDNA 4922502B01 gene	99.6	45.9	0.44
1441107_at	BB292639	Mm.32825	doublesex and mab-3 related TF like family A2	22	14.9	0.44
1444687_at	BB804635	Mm.337409	complement component 1, q subcomponent-like 2	56.4	26.3	0.44
1424865_at	BC010821	Mm.46248	peptide YY	35	12.4	0.47
1425288_at	BC026991	Mm.477583	sterile alpha motif domain containing 11	85.8	43	0.47
1427004_at	BB311718	Mm.262287	F-box protein 2	26.8	11.9	0.47
1450680_at	NM_009019	Mm.828	recombination activating gene 1	44.8	20.1	0.47
1450946_at	BG298773	Mm.20422	neural retina leucine zipper gene	632.8	289	0.47
1429945_at	AK013012	Mm.153585	kelch-like 35 (Drosophila)	27.9	15.1	0.47
1442120_at	AA153229	---	RIKEN cDNA G730007D18 gene	45.6	19.2	0.47

B

Probe-set	Accession No.	Unigene	Gene	WT signal	KO signal	Fold change
1439113_at	BB379753	Mm.327413	RIKEN cDNA 2410018L13 gene	24.2	91.1	4.9
1427820_at	BC021831	Mm.466733	---	47.4	159.7	4.0
1415801_at	M63801	Mm.378921	gap junction protein, alpha 1	39.2	132.6	3.4
1422674_s_at	NM_007775	Mm.440053	crystallin, gamma B /// crystallin, gamma C	24.2	88.3	3.0
1436115_at	BB829749	Mm.81916	predicted gene 266	28.6	87.3	3.0
1437726_x_at	BB111335	Mm.2570	complement component 1, q subcomponent, beta	35.7	96	3.0
1429948_x_at	AK013953	Mm.275362	crystallin, gamma F	20.7	95.6	3.0
1436127_at	AI854101	Mm.316614	corticotropin releasing hormone binding protein	156.9	474.7	3.0
1422652_at	NM_007774	Mm.26904	crystallin, gamma A	25.8	93.7	2.8
1449401_at	NM_007574	Mm.439732	complement component 1, q subcomponent, C chain	26.5	67.3	2.8
1426314_at	BB770914	Mm.229532	endothelin receptor type B	53.5	157.7	2.8
1450796_at	NM_016864	Mm.228661	atonal homolog 7 (Drosophila)	179.6	419.2	2.6
1417381_at	NM_007572	Mm.439957	complement component 1, q subcomponent, alpha	33.8	85.6	2.6
1446950_at	BM124834	Mm.87051	thymocyte selection-associated HMG box gene	23.8	60	2.6
1456659_at	BM116906	---	hypothetical LOC552902	27.2	69.2	2.6
1458432_at	BE948993	Mm.447891	non-catalytic region of tyrosine kinase adaptor protein 2	21.5	57.6	2.6
1423754_at	BC010291	Mm.141021	interferon induced transmembrane protein 3	25.2	65.7	2.6
1443337_at	BB531021	Mm.448265	glutamate receptor interacting protein 1	22.5	46.5	2.6
1445874_at	BB470735	Mm.439301	---	72.1	208	2.6
1438754_at	AV372127	---	---	26.8	69.1	2.6
1431335_a_at	AK018575	Mm.87599	WAP four-disulfide core domain 1	44.2	120.6	2.6
1460208_at	NM_007993	Mm.271644	fibrillin 1	44.4	82.1	2.4
1445561_at	BM218868	Mm.413457	---	25.6	56.4	2.4
1421141_a_at	BG962849	Mm.234965	forkhead box P1	22.2	60.4	2.4
1427183_at	BC023060	Mm.44176	EGF-containing fibulin-like extracellular matrix protein 1	85.6	253.9	2.4
1441577_at	BB419140	---	RIKEN cDNA C530014P21 gene	22.2	29.9	2.4
1437165_a_at	BB250811	Mm.262345	procollagen C-endopeptidase enhancer protein	24.8	64.9	2.4
1436905_x_at	BB218107	Mm.271868	lysosomal-associated protein transmembrane 5	26.7	64.7	2.4
1452892_at	AK014819	Mm.389950	serine/threonine kinase 33	23.3	52	2.4
1453145_at	AK007420	Mm.247625	phosphatidylserine decarboxylase, pseudogene 3	47	112.2	2.4
1417643_at	NM_025290	Mm.12743	radial spoke head 1 homolog (Chlamydomonas)	92.7	215.2	2.3
1448591_at	NM_021281	Mm.3619	cathepsin S	113.8	196.2	2.3
1421375_a_at	NM_011313	Mm.100144	S100 calcium binding protein A6 (calcyclin)	33.6	68.7	2.3
1449470_at	NM_010053	Mm.475101	distal-less homeobox 1	32.2	62.2	2.3
1458721_at	BG073332	Mm.247203	Protocadherin gamma cluster	24.3	50.3	2.3
1426607_at	BG068672	Mm.46679	predicted gene 7120	86.4	171.7	2.3

1460133_at	BF607205	Mm.450032	ephrin A5	21.8	61.4	2.3
1459731_at	BE996194	---	---	25.3	63.7	2.3
1446708_at	BB486740	Mm.135110	hypoxia inducible factor 3, alpha subunit	29.2	63.8	2.3
1443175_at	BB466434	---	RIKEN cDNA A830010M09 gene	22.9	66.3	2.3
1444345_at	AW123227	Mm.463057	---	23	55.9	2.3
1457111_at	AV318727	---	expressed sequence AA415038	26.9	55.9	2.3
1421011_at	NM_053262	Mm.46019	hydroxysteroid (17-beta) dehydrogenase 11	27	63.7	2.1
1418455_at	NM_019877	Mm.22144	coatomer protein complex, subunit zeta 2	27.6	55.9	2.1
1419665_a_at	NM_019738	Mm.18742	nuclear protein 1	49.2	120.1	2.1
1422903_at	NM_010745	Mm.2639	lymphocyte antigen 86	38	71.1	2.1
1417962_s_at	NM_010284	Mm.3986	growth hormone receptor	63.3	169	2.1
1448877_at	NM_010054	Mm.3896	distal-less homeobox 2	25.1	47.1	2.1
1421385_a_at	NM_008663	Mm.1403	myosin VIIA	38.4	79.2	2.1
1417346_at	BG084230	Mm.24163	PYD and CARD domain containing	37.9	76.2	2.1
1444349_at	BG071091	---	---	33.6	72.7	2.1
1452114_s_at	BF225802	Mm.405761	insulin-like growth factor binding protein 5	157.7	331.4	2.1
1442109_at	BE989344	Mm.445031	hypoxia inducible factor 3, alpha subunit	31.7	61.1	2.1
1422882_at	BE333485	Mm.246304	synaptophysin-like protein	24.4	58	2.1
1417625_s_at	BC015254	Mm.6522	chemokine (C-X-C motif) receptor 7	46.1	104.1	2.1
1450020_at	BC012653	Mm.44065	chemokine (C-X3-C) receptor 1	26.3	39.5	2.1
1452141_a_at	BC001991	Mm.392203	selenoprotein P, plasma, 1	212.5	484.9	2.1
1436098_at	BB667452	Mm.250719	butyrylcholinesterase	21.1	47.4	2.1
1436714_at	BB297498	Mm.209385	LIM domain containing preferred translocation partner in lipoma	43.2	82.9	2.1
1458408_at	BB160137	Mm.102765	sterile alpha motif domain containing 8	28.9	59.8	2.1
1435888_at	AV369812	Mm.420648	epidermal growth factor receptor	62.2	125.8	2.1
1456312_x_at	AV224521	Mm.21109	gelsolin	23.8	72.2	2.1