Top2b is Involved in the Formation of Outer Segment and Synapse During Late-stage Photoreceptor Differentiation by Controlling Key Genes of Photoreceptor Transcriptional Regulatory Network

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Topoisomerase II beta (Top2b) is an enzyme that alters the topologic states of DNA during transcription. Top2b deletion in early retinal progenitor cells causes severe defects in neural differentiation and affects cell survival in all retinal cell types. However, it is unclear whether the observed severe phenotypes are the result of cell-autonomous/primary defects or non–cell-autonomous/secondary defects caused by alterations of other retinal cells. Using photoreceptor cells as a model, we first characterized the phenotypes in Top2b conditional knockout. Top2b deletion leads to malformation of photoreceptor outer segments (OSs) and synapses accompanied by dramatic cell loss at late-stage photoreceptor differentiation. Then, we performed mosaic analysis with shRNA-mediated Top2b knockdown in neonatal retina using in vivo electroportation to target rod photoreceptors in neonatal retina. Top2b knockdown causes defective OS without causing a dramatic cell loss, suggesting a Top2b cell-autonomous function. Furthermore, RNA-seq analysis reveals that Top2b controls the expression of key genes in the photoreceptor gene–regulatory network (e.g., Crx, Nrx2e3, Opn1sw, Vsx2) and retinopathy-related genes (e.g., Abca4, Bbs7, Pde6b). Together, our data establish a combinatorial cell-autonomous and non–cell-autonomous role for Top2b in the late stage of photoreceptor differentiation and maturation.

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INTRODUCTION

Rod and cone photoreceptors constitute the largest cell population in the mammalian retina (Carter-Dawson and LaVail, 1979; Brzezinski and Reh, 2015; Cepko, 2015). A recent study using single-cell RNA-seq profiling has determined that the percentages of rods and cones in the mouse are 65.6% and 4.2%, respectively (Macosko et al., 2015). Lineage studies showed that both cell types are derived from multipotent retinal progenitor cells (reviewed by Brzezinski and Reh, 2015; Cepko, 2015; Wang and Cepko, 2016). Birth-dating studies have established that the genesis of retinal cell types is a highly

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Topoisomerase II beta (Top2b) is an enzyme that controls and alters the topologic states of DNA during transcription (Wang, 2002). The onset of Top2b expression is observed in progenitor cells that just finished the final division and is actively involved in neural development (Tsutsui et al., 1993; Tsutsui et al., 2001; Lyu and Wang, 2003; Tiwari et al., 2012). Many studies have established a multifaceted role of Top2b in 1) resolving the topological constraints in early-stage neuronal gene expression (Madabhushi et al., 2015); 2) neurite guidance during late-stage neuronal development (Yang et al., 2000; Nevin et al., 2011); 3) cerebral stratification (Lyu and Wang, 2003); and 4) control of developmentally regulated genes in the brain and neural retina (Lyu et al., 2006). We have shown severe defects in differentiation and survival of retinal neurons and Müller glia in Dkk3-Cre-mediated Top2b conditional knockout animals (cKO) (Li et al., 2014). However, because of the pleiotropic effect of Top2b knockout on gene expression, the mechanism underlying Top2b functions (e.g., cell-autonomous (primary) or non–cell-autonomous (secondary)) during retinal development is unclear.

In this study, we show that Top2b plays an important role in photoreceptor development in both a cell-autonomous and non–cell-autonomous manner. Transcriptome analysis by RNA-seq reveals that Top2b controls key genes in the photoreceptor regulatory network and genes linked to retinopathies.

**MATERIALS AND METHODS**

**Sample Collection**

All studies performed were conducted under the strict guidelines and consent of the Institutional Animal Care and Use Committee of Rutgers, the State University of New Jersey. Top2b cKO animals were generated by breeding a strain of Dkk3-Cre mice (Sato et al., 2007) with the Top2b-floxed mice on a 129SvEv background. Three to five animals from each genotype were used for each stage. No animal was excluded. Gender of the animals was not reported because no difference between different sexes was observed. Animals were euthanized with CO2 inhalation. For immunohistochemistry, retinai were dissected from the animals and fixed with 4% (w/v) PFA for 1 hr. Following fixation, samples were washed three times with 1 × PBS for 10 min each, soaked in 30% (w/v) sucrose for 2 to 3 days, embedded in cryopreserving media (Tissue Tek® OCT compound, SAKURA FINETEK USA INC, Torrance, CA), and stored at −80°C. Cryosectioning was performed with a cryostat (Thermo Scientific, Waltham, MA). Sections of samples were air dried for 10 min and stored at −80°C. For RNA-seq analysis, P0 and P6 retinae (n ≥ 4 for each genotype on each stage) were dissected rapidly free of other ocular tissue and frozen with liquid nitrogen. For total RNA isolation, retinal samples were transferred into 500 µl of TRIzol reagent (Invitrogen), and isolation was performed according to the manufacturer’s instructions.

**Immunohistochemistry**

Immunofluorescence staining was performed on mouse eye sections as described previously (Li et al., 2014). The primary antibodies used in this study are listed in Table I. Images were captured using a Zeiss Axio Imager M1 fluorescence microscope and analyzed using AxioVision 4.8 (Zeiss, Germany). For proper comparison between control and cKO samples, all images were captured from the dorsal–medial region of the retina. Cell counting and measurements were performed in the central region of the retina, and the regions were randomly selected. For each data point, samples from at least three animals were calculated for an average and standard deviation. Two-tailed heteroscedastic Student t-test was employed to calculate P values.

**Helium Ion Microscopic Imaging**

Retinai from control and Top2b cKO mice were collected, fixed, and sectioned as described previously (Li et al., 2014). Each slide contains 8 to 10 sections, which are ∼10 to 12 µm thick. For chemical drying, the slides were subjected to sequential dehydration in graded ethanol (Fisher Scientific, Waltham, MA) (50%, 70%, 80%, 90%, and 100%) baths at 20-minute intervals. After the final 20 minutes of ethanol dehydration, solutions of different ratios of ethanol and hexamethyldisilazane (HMDS, UltraPure Solutions, Inc., Castroville, CA) (25%, 50%, 75%, 100%) were added sequentially to replace the inner-tissue solution from ethanol to HMDS. The tissue samples were removed from the 100% HMDS bath, placed in a fume hood with cover for 12 hr, and stored in a newly charged desiccation chamber under vacuum. For helium ion microscopy (HIM; ORION Plus, Carl Zeiss, Peabody, MA), the sample
was mounted onto an HIM stub with carbon tape, and images were taken with different fields of view using ORION PLUS software.

Cre-mediated Top2b Knockout and shRNA-mediated Top2b Knockdown

For Top2b gene knockout in postnatal mouse retinae, a CAG-Cre-GFP construct (Addgene plasmid #13776) was injected into the subretinal space of P0 pups of Top2bf/f mice (Lyu and Wang 2003). For Top2b knockdown, an effective Top2b shRNA (shTop2b) and a control plasmid construct using LentiLox 3.7 vector (Azarova et al., 2010) were used. Plasmid DNA injection and electroporation were performed following a published protocol (Matsuda and Cepko, 2004). Briefly, the plasmids were mixed with FastGreen (0.025%), and 0.5 ml of DNA was injected into the subretinal space between the retina and choroid of P0 wild-type mouse pups. After injection, tweezer-type electrodes (model 520; 7-mm diameter; BTX, San Diego, CA) briefly soaked in PBS were placed to softly hold the heads of the pups, and five 80V square pulses of 50-msec duration with 950-msec intervals were applied using a pulse generator ECM830 (BTX, San Diego, CA). Usually DNA was transfected only into right eyes. Transfected retinae were harvested at various stages and sectioned for immunostaining as described (Li et al., 2014).

RNA-seq Analysis

Whole-transcriptome RNA libraries were sequenced using the SOLiD System (Applied Biosystems). Data analysis was performed according to published protocols (Trapnell et al., 2012) with minor modifications. Briefly, colorspace data of raw 50-bp reads were aligned to the mouse genome (mm9 or GR.Cm37) using Bowtie (Langmead et al., 2009). Gene expression levels were analyzed with Cufflinks, with differentially expressed genes determined by Cuffdiff (Trapnell et al., 2012). Full raw and processed data have been deposited to NCBI with GEO accession number GSE86187. Coverage plots were generated using BEDTools (http://code.google.com/p/bedtools/) with BEDGraph and BigWig files, which were then visualized in the UCSC Genome Browser (http://genome.ucsc.edu).

Ingenuity Pathway Analysis

Top2b-dependent genes (TDGs) (fold change ≥ 1.5; P value ≤ 0.05) were determined using Cuffdiff (Trapnell et al., 2012) and further analyzed using Ingenuity Pathway Analysis tools (www.qiagen.com/ingenuity). Significance of the three output panels (i.e., Biological process, Pathway, Disease) was calculated using right-tailed Fisher exact test.

Analysis of TDGs With Gene Ontology Database and Disease Databases

TDGs related to neuronal cell death, neurite outgrowth, and visual system development were analyzed with open-access databases including Gene Ontology (http://www.geneontology.org), multiple retinal disease databases (RetNet, https://sph.uth.tmc.edu/Retnet/; Disease Atlas; NextBio, http://www.nextbio.com), and mitochondrial disease–related gene lists (Yu-Wai-Man et al., 2011) for their functions and disease-related features.

RESULTS

Top2b Deficiency Causes Developmental Defects in Photoreceptor Outer Segment Formation

In our previous study, we showed that Top2b cKO (Top2b<sup>−/−</sup>; DKK3-Cre) in early retinal progenitor cells leads to defects in differentiation and survival of all retinal cell types
(Li et al., 2014). In this study, we attempt to define the molecular mechanism of Top2b function using the photoreceptor cell as a model. First, retinal samples from the cKO (Top2b<sup>−/−</sup>) and control (Top2b<sup>+/−</sup> and Top2b<sup>+/+</sup>) mice at various stages were analyzed by immunostaining with rod marker rhodopsin (Fig. 1A), s-cone marker Opn1sw (Fig. 1B), and m/l-cone marker Opn1mw/lw (Fig. 1C). Rhodopsin expression was detected only in a few cells in the outer nuclear layer (ONL) of both the control and cKO samples starting at E19.5, and became extensive at P7 and P14 (Fig. 1A). At P7 and P14, noticeably thinner ONLs were observed in the cKO retina (Fig. 1A, D), and there was no or little rhodopsin accumulation near the outer limiting membrane (purple arrows in Fig. 1A). By P14, rhodopsin signal was not detected or significantly reduced in the OS with a strong signal in the cell bodies (Fig. 1A, E). In addition, Opn1sw-labeled s-cones showed dramatic degeneration at P0, P7, and P14 (Fig. 1B). Opn1sw + cells were observed with shorter processes at P0 (arrowheads in Fig. 1B). No neural process was detected at P7 and P14 (Fig. 1B). The OS was only observed in control samples (purple arrows in Fig. 1B) but not in cKO samples. By P14, the vast majority of Opn1sw + s-cones were degenerated in the cKO retinae (Fig. 1B, F). Moreover, examination of the m/l-cones by anti-Opn1mw/lw antibody staining showed that in the cKO opsins were accumulated in small regions, but no OS can be identified (purple arrows in Fig. 1C). A strong signal of Opn1mw/lw was found in cytoplasm (yellow arrows in Fig. 1C). It is also noted that the rhodopsin- and Opn1mw/lw-labeled ONL cells intruded into the inner nuclear layer (INL) in P14 cKO samples (Fig. 1A, C).

To further determine the defects in the OS at a micrometer ultrastructural level, we examined retinal sections using HIM (Zeiss, Germany). In both P14 and P21 control retinae, the OSs of both rod and cone cells were found between the retinal pigment epithelial cell (RPE) layer and the ONL (arrows in Fig. 1G). In contrast, there was no distinguishable OS structure in cKO retinae at these two stages (Fig. 1G). In addition, the retina in cKO samples was largely detached from the RPE.

**Aberrant Synapse Formation in Top2b cKO Retinae**

Although we reported missing outer plexiform layer (OPL) in our previous study (Li et al., 2014), it was not clear whether it was due to the absence of horizontal cells or interruption of synaptic connections between photoreceptor cells and bipolar cells. The observed phenotypes in the OS lead us to speculate that axonal/dendritic growth and synapse formation of photoreceptor cells may be affected in the cKO mouse retinae. Therefore, retinal sections of the control and cKO animals were stained with antibodies against vGlut1 (a vesicular glutamate transporter) (Fig. 2A) and synaptophysin (a synaptic vesicle protein) (Fig. 2B). vGlut1 is required for synaptic vesicles with glutamate at the synaptic terminals (Johnson et al., 2003) and is usually found in the presynapses (Sherry et al., 2003). Expression of vGlut1 in control retinae suggested the extension of photoreceptor cell synapse into OPL at P7 and formation of bulb-shaped structures at P14 and P21 (arrowheads in Fig. 2A). In cKO retinae, however, vGlut1 was expanded in the cytoplasm, and no bulb-shaped structure can be found (Fig. 2A). In addition, synaptophysin is expressed in all vesicular synaptic terminals in both the OPL and inner plexiform layer (IPL) of the retina (Brandstatter et al., 1996; Spiwoks-Becker et al., 2001). Synaptophysin signals were prominent in the synapses within the OPL (dotted lines in Fig. 2B) at P7, P14, and P21. In the cKO, synaptophysin signals at P14 and P21 became more prominent in the cytoplasm of the ONL cells, and the OPL structure was disrupted (Fig. 2B).

**Increased Photoreceptor Cell Death in the ONL of Top2b cKO**

Defects in axonal/dendritic growth and the formation of synapse and OS usually lead to photoreceptor degeneration (Swaroop et al., 2010). Thus, we analyzed photoreceptor cell death by immunostaining with apoptosis marker–activated caspase 3 (Casp3; Fig. 3A). Compared with the control, a significantly increased number of Casp3+ cells were observed in the ONL of cKO retinae at P7, P14, and P21 (Fig. 3B).

**Cell-autonomous Role of Top2b in Rod OS Formation by Mosaic Cell Analysis**

Because the cKO animals showed defects in almost all retina cell types (Li et al., 2014), it is unclear whether the observed defects in photoreceptors were due to cell-autonomous or non–cell-autonomous effects. To address this issue, we performed a mosaic analysis by targeted Top2b knockout in postnatal rod photoreceptor cells (rods) by injection and electroporation of plasmid construct CAG-Cre-GFP into the eye in P0 Top2b<sup>−/−</sup> animals (Lyu and Wang, 2003). Animals were harvested and analyzed at P7. We found that almost all GFP+ cells (Top2b knockout) were rods and located in the INL, while the majority of DsRed+ rods (control) were in the ONL (Fig. S1), a phenotype of delayed cell differentiation. In addition, no significant cell death was detected in GFP+ cells (Fig. S1C). These results support a cell-autonomous role of Top2b in rod cell differentiation. To further confirm this cell-autonomous function of Top2b, shRNA-mediated gene silencing analysis was performed. Plasmid DNA shTop2b-GFP was injected and electroporated into P0 retina of the wild-type mice. Transfected retinae were examined at P14 and P21 after P0 electroporation (Fig. 4A). Transfection led to strong reporter GFP expression in rods in the ONL (Fig. 4B). The number of GFP+ cells was counted, and the percentage of GFP+ cells in the total number of ONL cells was determined (Fig. 4C). The efficiency of Top2b knockdown was evaluated by immunostaining analysis with anti–Top2b antibody, which showed a reduction or elimination of Top2b expression in the transfected cells (arrows in Fig. 4B). Over 97% of GFP+ cells at P7 and almost 100% at both P14 and P21 were negative for Top2b staining (Fig. 4C), indicating a successful gene knockdown. Comparing...
Fig. 1. Top2b deletion leads to malformation of outer segment structure in both rod and cone cells. OS structure in retinal sections was examined by immunostaining with rod marker rhodopsin (A), s-cone marker Opn1sw (B), m/l-cone marker Opn1mw/lw (C), and helium ion microscopy (HIM) (G). In Top2b cKO, there was a significant reduction of OS thickness in all photoreceptor cells. OS structure was mostly missing or barely identifiable in HIM. **Purple arrows** indicate the OS, **red arrowheads** indicate the processes of photoreceptor cells, and **yellow arrows** indicate marker expression inside cell bodies. Images in boxed areas are shown in a higher magnification. The thickness of the ONL and OSL of rod cells was measured as indicated by the bar lines and plotted (D and E). The number of s-cone cells was counted and plotted (F). OLM, outer limiting membrane; ONL, outer nuclear layer; INL, inner nuclear layer; GCL, ganglion cell layer; OS, outer segment; OSL, outer segment layer; RPE, retinal pigment epithelium. Scale bar in panel A-C = 50/10 (enlarge) μm; G = 5 μm. Data in plots are represented as median ± max/min (n ≥ 3). P values were determined by Student t-test.
shTop2b knockdown samples with the controls (scrambled shRNA), there was a significant reduction of OS length (Fig. 4F, bars and chart) and disorganized OS (Fig. 4F, white arrows) in transfected GFP cells at P21. In contrast to the dramatic phenotypes observed in cKO animals, there was no significant difference of ONL thickness, ONL cell number (Fig. 4E), or synapses (yellow arrows, Fig. 4F), indicating that Top2b knockdown did not cause a dramatic cell loss. Thus, these results further support a cell-autonomous role of Top2b in rod cell differentiation.

Top2b Deficiency Affects the Expression of Key Genes in a Transcriptional Network for Photoreceptor Differentiation and Maintenance

Top2b is known to regulate gene transcription (Wang, 2002). To determine genes controlled by Top2b, we performed transcriptome analysis using RNA-seq data from control and cKO retinae at P0 and P6. The two time points (i.e., P0 and P6) were selected because 1) P0 is at the peak of rod photoreceptor genesis and cone maturation, 2) P6 is at the middle stage of rod differentiation, and 3) rods comprise the vast majority (94%) of the photoreceptor cell population (Young, 1985a; Brzezinski and Reh, 2015). Thus, sequencing results for these two stages largely represent the genes required for photoreceptor cell differentiation and maturation. Four libraries were constructed: P0 control, P0 cKO, P6 control, and P6 cKO (Table S1). Differentially expressed genes contrasting the control and Top2b cKO samples were determined using Cufflinks and Cuffdiff (Trapnell et al., 2010, 2012). We identified 699 genes at P0 and 180 genes at P6 (Fig. 5A, B; Table S2, S3) that were significantly changed in expression level (\( |\log_{2} \text{fold change}| \geq 1.5 \), \( P \text{ value} \leq 0.05 \)), including Top2b itself (Fig. 6A). These differentially expressed genes are thus referred to as the Top2b Dependent Genes (TDGs). No significant change was detected in the expression of 20 known housekeeping genes (Table S4), confirming that the sequencing and calculation of transcript abundances was of good quality.

To explore the role of Top2b in photoreceptor development, TDGs were analyzed by Ingenuity Pathway Analysis (www.ingenuity.com, Redwood City, CA, USA), a web-based tool for statistical analysis and biological interpretation of gene expression, in parallel with analysis via literature review and functional classification from the Gene Ontology Consortium. Top2b-dependent interactions, pathways, and disease-related gene pools were
obtained to predict its regulatory role. Interestingly, the analysis reveals six TDGs (i.e., Crx, Nr2e3, Vsx2, Opn1sw, Glo1, and Smim13) (Table II) that are critical for rod and cone photoreceptor cell fate determination, differentiation, and maturation (Furukawa et al., 1997; Hennig et al., 2008; Swaroop et al., 2010; Cepko, 2015). The altered expression level of Crx and Nr2e3 was shown in coverage plots (Fig. 6B, C). It is likely that Top2b modulates photoreceptor cell differentiation by regulating the transcription of these pivotal genes in the regulatory network (Fig. 6D).

Top2b-associated Genes Are Linked With Photoreceptor-related Retinopathy

The degeneration of photoreceptor cells causes many retinal diseases that ultimately lead to blindness (Clarke et al., 2000). To determine the association of Top2b with these retinal diseases, TDGs were searched against the databases of genes related to retinopathy (RetNet; NextBio Disease Atlas [Montana and Corbo, 2008]). Arl6, Atxn7, Bbs7, Crx, Dmd, Nr2e3, Pde6b, Pxmp3, Rbp4, Trpm1, Abca4, Iqcb1, Opn1sw, and Pgk1 were found to be linked to various retinal diseases—for instance, retinitis pigmentosa, S-cone syndrome, congenital stationary night blindness, and syndromes including retinopathies such as Bardet-Biedl syndrome (Table III).

DISCUSSION

Photoreceptors constitute the majority of the retinal cell population, and their development expands from...
embryonic to neonatal stages (Young, 1985a,b; Rapaport et al., 2004; Brzezinski and Reh, 2015; Wang and Cepko, 2016). In this study, we report a combinatorial cell-autonomous and non–cell-autonomous role of Top2b during late-stage photoreceptor differentiation and maturation.

Top2b Functions in the Late-stage Differentiation and Maintenance of Photoreceptor Cells

Dkk3-Cre–mediated cKO resulted in Top2b deletion in retinal progenitor cells and all progeny in all retinal cell types (Li et al., 2014), making it a valuable approach to study Top2b function in the development of all retinal cell lineages including photoreceptors. In Top2b cKOs, both cones and rods were generated but failed to fully differentiate. Deficits exist in i) the OS maturation and maintenance (Fig. 1), ii) accumulated opsin in the cell body (Fig. 1A–C), iii) synapse formation (Fig. 2), and iv) cell degeneration and cell death in both rods and cones (Fig. 3). The defects in the OS with traditional fluorescence microscopy were confirmed by HIM. HIM provides a new approach to investigate nanometer-scale surface features of biological samples.
(Joens et al., 2013). With this technique, detailed differences on surface ultrastructure of photoreceptors between the control and cKO mice were revealed clearly (Fig. 1G). Similar defects were also observed in late-stage development in the animals lacking Crx (Furukawa et al., 1999), Nrl (Daniele et al., 2005), or Vsx2 (Rutherford et al., 2004), except that in these animals there was an increase in the number of cone cells. In addition, the lack of OS and the degeneration of photoreceptor cells are usually observed in the opsin-deficient animals (Daniele et al., 2011; Lem et al., 1999). It is believed that mislocalized opsin is frequently associated with retinal degeneration (Deretic, 2006; den Hollander et al., 2008) and has been indicated as a major cause of photoreceptor cell death in the absence of heterotrimeric kinesin-2 function (Louie et al., 2010). The enriched rhodopsin and Opn1sw/mw/lw in photoreceptor cell bodies of cKO retinae (Fig. 1A–C) may explain the dramatic cell loss found in the ONL (Fig. 3). Because cone cells represent 3% to 5% of all photoreceptor cells, the dramatic cell loss observed in the ONL is most likely due to the rod cell phenotype.

Cell-autonomous and Non–cell-autonomous Role of Top2b in Photoreceptor Differentiation

Dkk3-Cre–mediated Top2b cKO provides valuable insight into retinal development. However, it has limitations for addressing cell-autonomous vs. non–cell-autonomous and primary vs. secondary effects of Top2b on photoreceptor differentiation. Thus, we performed mosaic analysis using shRNA-mediated Top2b knockdown in postnatal developing photoreceptor cells. Shorter OS was observed in the sample transfected with shTop2b (Fig. 4F) without dramatic cell loss (Fig. 4D), suggesting that a cell-autonomous function of Top2b is primarily affecting OS maturation and maintenance. Moreover, in contrast to a dramatic synapse degeneration (Fig. 2), cell degeneration, and cell death (Fig. 3) observed in the cKO, Top2b knockdown...
did not cause a noticeable difference in the thickness of the ONL or morphology of synapses (Fig. 4E, F). Cell-autonomous effects alone cannot account for the severe phenotypes observed in the retinae of cKO mice. Therefore, the multilevel defects in photoreceptor differentiation in cKO animals are likely due to both cell-autonomous (primary) and non–cell-autonomous (secondary) effects of Top2b. As photoreceptors directly form synaptic connections with the horizontal and bipolar cells, alterations in these two cell types usually lead to defects in photoreceptor cells. A study has shown that the loss of horizontal cells leads to partial rod photoreceptor cell degeneration, retraction of axons from the OPL, and partial cell death (Sonntag et al., 2012). Because defects of the horizontal cells but

**Fig. 6.** Top2b regulates key genes in the photoreceptor transcriptional network. Several key genes in the transcriptional network for photoreceptor development were recognized as Top2b-dependent genes (TDGs) by RNA-seq analysis. Coverage plots of Top2b (A) Crx (B), and Nr2e3 (C) show differential gene expression between control and Top2b cKO samples. The number of sequence reads mapped to their genes is plotted on their genomic locations. The maps of alternative spliced transcripts of these genes are also shown underneath the plot. (D) Schematic drawing of photoreceptor transcriptional network with Top2b as key modulator. Names of the downregulated genes are shown in blue; upregulated gene Vsx2 is in orange. Magenta lines indicate the effect of Top2b on those key genes.

**TABLE II.** List of Top2b-dependent Genes Critical for Photoreceptor Cell Development Identified by RNA-Seq

| Gene symbol | WT FPKM | cKO FPKM | Log2 (fold change) | P value |
|-------------|---------|----------|-------------------|---------|
| P0 Crx      | 61.41   | 26.66    | −1.21             | 3.26E-06|
| Nr2e3       | 21.04   | 5.95     | −1.82             | 2.57E-06|
| Glo1        | 80.36   | 34.34    | −1.23             | 1.84E-06|
| Smim13      | 8.56    | 3.66     | −1.22             | 0.00187 |
| Vsx2        | 11.49   | 26.76    | 1.22              | 4.86E-04|
| P6 Opn1sw   | 29.47   | 11.45    | −1.36             | 3.16E-12|
| Glo1        | 194.61  | 107.153  | 0.86              | 3.27E-07|

*Note:* FPKM, fragments per kilobase of transcript per million mapped reads.
not the bipolar cells were observed in Top2b cKO animals (Li et al., 2014), it is likely that horizontal cell degeneration exacerbates the photoreceptor phenotype. However, in the same study (Sonntag et al., 2012), the s-cones remained largely unchanged, which is different from our observation of a dramatic s-cone cell loss (Fig. 1B). It will be intriguing to find out what is responsible for the s-cone dystrophy.

Surprisingly, synapse formation was not affected in the mosaic retinae with shTop2b knockdown (Fig. 4F), suggesting that Top2b may not play a cell-autonomous role in this process. Top2b is known to be important for axon guidance and neurite growth of motor neurons (Yang et al., 2000). We also found reduced optic nerve and disrupted IPL and OPL in cKO retina (Li et al., 2014). These observations suggest that Top2b might play a different role among various types of neurons.

### TABLE III. Differential Expression of Retinopathy-related Genes in Top2b cKO Retinae

| Stage | Gene  | Log2 (fold change) | P value | Q value | Disease                                                                 | Reference                                                                 |
|-------|-------|--------------------|---------|---------|-------------------------------------------------------------------------|---------------------------------------------------------------------------|
| P0    | Arl6  | -2                 | 0.00161861 | 0.030496 | Recessive Bardet-Biedl syndrome                                          | (Chiang et al., 2004; Fan et al., 2004)                                     |
|       | Atxn7 | -2                 | 0.00299047 | 0.048919 | Dominant spinocerebellar ataxia with macular dystrophy or retinal degeneration | (Aleman et al., 2002)                                                    |
|       | Bbs7  | -4.1               | 0.000536956 | 0.012973 | Recessive Bardet-Biedl syndrome                                          | (Badano et al., 2003)                                                    |
|       | Crx   | -1.2               | 3.25587E-06 | 0.000281 | Dominant cone-rod dystrophy; recessive, dominant and de novo Leber congenital amaurosis; dominant retinitis pigmentosa | (Furukawa et al., 1999; Hanein et al., 2004; Menotti-Raymond et al., 2010) |
|       | Dmd   | -1.5               | 0.000626471 | 0.014728 | Oregon eye disease (probably)                                            | (D’Souza et al., 1995)                                                   |
|       | Nr2e3 | -1.8               | 2.56724E-06 | 0.000241 | Recessive enhanced s-cone syndrome; recessive retinitis pigmentosa in Portuguese Crypto Jews; Goldmann-Favre syndrome; dominant retinitis pigmentosa; combined dominant and recessive retinopathy | (Coppieters et al., 2007; Escher et al., 2009; Sharon et al., 2003) |
| P6    | Pmp3  | -1.4               | 0.000157789 | 0.005179 | Recessive Refsum disease, infantile form                                  | (Gartner et al., 1992)                                                   |
|       | Trpm1 | -4.3               | 8.81098E-06 | 0.000589 | Recessive congenital stationary night blindness, complete                | (Audo et al., 2009)                                                     |
|       | Pde6b | 1.93               | 0.00273468 | 0.045837 | Recessive retinitis pigmentosa; dominant congenital stationary night blindness | (Riess et al., 1992)                                                   |
|       | Rbp4  | 5.5                | 0.000100318 | 0.003672 | Recessive RPE degeneration                                               | (Seeliger et al., 1999)                                                  |
|       | Iqcb1 | < -1024            | 3.03261E-11 | 2.15792E-08 | Recessive Senior-Loken syndrome; recessive Leber congenital amaurosis       | (Estrada-Cuzcano et al., 2011; Otto et al., 2005) |
|       | Opn1sw| -1.4               | 3.15836E-12 | 2.5511E-09 | Dominant tritanopia                                                       | (Weitz et al., 1992)                                                    |
|       | Pkg1  | -1.1               | 2.70792E-08 | 1.09363E-05 | Retinitis pigmentosa with myopathy                                       | (Tonin et al., 1993)                                                    |
|       | Abca4 | 1.71               | 1.77636E-15 | 1.90E-12   | Recessive Stargardt disease, juvenile and late onset; recessive macular dystrophy; recessive retinitis pigmentosa; recessive fundus flavimaculatus; recessive cone-rod dystrophy | (Gerber et al., 1995; Maugeri et al., 2000; Molday et al., 2000) |

*Note: Genes involved in photoreceptor cell-related retinal diseases are shown in bold.*

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Top2b Controls Key Genes in the Photoreceptor Gene-regulatory Network

Rods constitute the majority of the cells in the mouse retina (Carter-Dawson and LaVail, 1979; Brzezinski and Reh, 2015; Cepko, 2015). TDGs (e.g., Vsx2, Crx, Nr2e3, Opn1sw, Glo1, Smin13) identified by RNA-seq analysis (Figs. 5 and 6) are key genes in the regulatory network that dictates photoreceptor differentiation and homeostasis. Thus, Top2b functions as a key modulator at multiple levels in this hierarchical transcriptional regulatory network (Fig. 6E). Among the six photoreceptor-related TDGs, Vsx2 mutation leads to delayed Crx expression and rod development (Rutherford et al., 2004). Crx and Nr2e3 are known to be essential genes in controlling the rod/cone cell fate determination (Peng and Chen, 2005; Peng et al., 2005) and development of the OS (Furukawa et al., 1997). These studies are consistent with the observed deficits in photoreceptor cells in Top2b cKO animals, except that...
mutation of Nr2e3 can lead to excess s-cones (Peng et al., 2005). It is possible that Top2b deletion affects Crx and Nr2e3 expression and consequently affects Opn1sw and rhodopsin expression at a later stage during photoreceptor differentiation. Alternatively, Top2b may directly regulate Opn1sw expression by binding to a distant regulatory region ~200 kb upstream of the Opn1sw transcription starting site (data from GSM1516578 in Madabhushi et al., 2015). As Opn1sw encodes the blue cone pigment (Nathans et al., 1986), the reduction in Opn1sw expression may also be a contributor to the dramatic loss of the OS and the number of s-cones in the cKO.

Several TDGs have been known to function in neurite growth and retinal cell maintenance including Igfl, Crefb, Hras, Mark1, Grin1, Cdkn1a, Serpine2, Ptpzr2, Gnao1, Drd2, Pdia3, and Dpysl3. Although neurite degeneration and neural cell death can be separate events (Ikegami and Koike, 2003), our observation favors the idea that the impaired neurite outgrowth accelerates retinal cell death.

Finally, mice lacking Top2b present a transcriptome profile that is prone to several photoreceptor-related retinal diseases such as retinitis pigmentosa, s-cone syndrome, and Bardet-Biedl syndrome (Table III). The identification of retinopathy-related TDGs suggests that Top2b function is required for not only normal development but also the prevention of retinal diseases.
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