Jmjd3 Plays Pivotal Roles in the Proper Development of Early-Born Retinal Lineages: Amacrine, Horizontal, and Retinal Ganglion Cells

Toshiro Iwagawa,[1] Hiroaki Honda,[2] and Sumiko Watanabe[1]

[1]Division of Molecular and Developmental Biology, Institute of Medical Science, University of Tokyo, Tokyo, Japan
[2]Field of Human Disease Models, Major in Advanced Life Sciences and Medicine, Institute of Laboratory Animals, Tokyo Women's Medical University, Tokyo, Japan

Correspondence: Sumiko Watanabe, Division of Molecular and Developmental Biology, Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan; sumiko@ims.u-tokyo.ac.jp.

Received: April 12, 2020
Accepted: September 14, 2020
Published: September 28, 2020

Citation: Iwagawa T, Honda H, Watanabe S. Jmjd3 plays pivotal roles in the proper development of early-born retinal lineages: Amacrine, horizontal, and retinal ganglion cells. *Invest Ophthalmol Vis Sci.* 2020;61(11):43. https://doi.org/10.1167/iovs.61.11.43

Purpose. Trimethylation of histone H3 at lysine 27 (H3K27me3) is a critical mediator of transcriptional gene repression, and *Jmjd3* and *Utx* are the demethylases specific to H3K27me3. Using an in vitro retinal explant culture system, we previously revealed the role of *Jmjd3* in the development of rod bipolar cells; however, the roles of *Jmjd3* in the development of early-born retinal cells are unknown due to limitations concerning the use of retinal explant culture systems. In this study, we investigated the roles of *Jmjd3* in the development of early-born retinal cells.

Methods. We examined retina-specific conditional *Jmjd3* knockout (Jmjd3-cKO) mice using immunohistochemistry and quantitative reverse transcription PCR and JMJD3 binding to a target locus by chromatin immunoprecipitation analysis.

Results. We observed reductions in amacrine cells (ACs) and horizontal cells (HCs), as well as lowered expression levels of several transcription factors involved in the development of ACs and HCs in the Jmjd3-cKO mouse retina. JMJD3 bound the promoter regions of these transcription factors. Notably, an elevated number of retinal ganglion cells (RGCs) was observed at embryonic stages, whereas RGCs were moderately reduced at later postnatal stages in the Jmjd3-cKO retina. We also observed reduced expression of *Eomes*, which is required for the maintenance of RGCs, as well as lower H3K27me3 level and lower JMJD3 binding in the promoter region of *Eomes* in RGC-enriched cells.

Conclusions. The results indicated that *Jmjd3* has critical roles in the development of early-born retinal subtypes, and suggested biphasic roles of *Jmjd3* in RGC production and maintenance.

Keywords: histone methylation, demethylase, H3K27, early retinal development

During retinal development, retinal progenitor cells (RPCs) proliferate and differentiate into six types of neurons and one type of glial cell. The temporal order of the production of each retinal cell is highly regulated and conserved among species.[1,2] The molecular mechanisms that determine cell fate and maturation have been extensively investigated, and the roles of transcription factors in retinal development have received considerable attention.[1,3–6] Moreover, there is increasing clarity regarding the contributions of epigenetic modifications (e.g., DNA methylation and histone modifications) to retinal development.[7–9]

Trimethylation of histone H3 at lysine 27 (H3K27me3) is a critical mediator of transcriptional gene repression and is related to many important biological processes, including cellular differentiation.[10] H3K27me3 is mainly regulated by methyltransferase polycomb repressor complex 2 (PRC2) and the demethylases *Jmjd3* (*Kdm6b*) and *Utx* (*Kdm6a*).[11] *Ezh2* (*Kmt6a*) is a core component of PRC2 that catalyzes H3K27me3.[12] In *Xenopus*, knockdown of *ezh2* has been shown to cause a deficiency in the proliferation of RPCs and an excess proportion of Müller cells, highlighting the role of Ezh2 in the proliferation and timing of neurogenesis.[13] Studies of conditional *Ezh2* knockout (Ezh2-cKO) mice revealed reductions in the proliferation of RPCs at early postnatal stages and showed accelerated onset of the differentiation of late-born retinal cells, including rods and Müller cells.[14,15] Notably, the upregulation of *Six1* in Ezh2-cKO mice caused accelerated rod differentiation.[16]

During retinal development in mice, *Jmjd3* is highly expressed at postnatal stages; immunohistochemical analyses have shown that JMJD3 is expressed in the inner nuclear layer (INL) and ganglion cell layer (GCL), but not in the outer nuclear layer.[17] To reveal the roles of *Jmjd3*, especially in the development of late-born retinal cells, we performed loss-of-function analysis of *Jmjd3* in an in vitro retinal explant; the results indicated that *Jmjd3* is involved in the development of subsets of retinal bipolar cells (BCs), rod-ON-BCs, and type II cone-OFF-BCs, through regulation of the expression of *Bhlhb4* and *Vsx1*, respectively.[17] Because the H3K27me3 levels of these target genes are high throughout

the retina, we propose that timely, spatially controlled derepression caused by the lineage-specific function of *Jmjd3* is critical for BC development; indeed, a subset of retinal cells including BCs showed lower H3K27me3 levels. This notion was supported by our ChIP-Seq analysis of H3K27me3 in retinal cells, which revealed that the H3K27me3 levels of cell-type-specific transcription factors are regulated in a cell-type-dependent manner.[18] However, examination of the Ezh2-cKO retina did not show simple augmentation of transcription factors, suggesting that H3K27me3 is a key mechanism that represses lineage-specific genes in a lineage-specific manner, whereas transcription activation is achieved by collaboration with other positive mechanisms.[14,15]

Although our previous study showed that *Jmjd3* is expressed from embryonic stages and strongly expressed in the postnatal GCL,[17] the *in vitro* retinal culture system used previously is technically unsuitable for the study of early embryonic retinal development. Therefore in this study we used a retina-specific *Jmjd3* conditional knockout (Jmjd3-cKO) mouse line, which showed knockout of *Jmjd3* from embryonic day 10 (E10) or earlier. We found that *Jmjd3* is critical for the development of amacrine cells (ACs), horizontal cells (HCs), and retinal ganglion cells (RGCs) at early stages and the maintenance of RGCs at late postnatal stages.

Methods

Mice

In *Jmjd3* floxed mice, exons 17–19 in the *Jmjd3* gene, which encode a part of the JmjC domain, are flanked by loxP sites (Fig. 2A). To generate retina-specific *Jmjd3* knockout (Jmjd3-cKO) mice, Utx-cKO mice, or *Jmjd3* and *Utx* conditional double knockout (Jmjd3/Utx-cDKO) mice, *Dkk3*-Cre mice, which express the Cre gene in retinal progenitor cells from embryonic day 10.5 (E10.5),[19] were crossed with *Jmjd3* floxed and *Utx* floxed mice.[20] The presence of a vaginal plug was defined as E0.5 and the day of birth as postnatal day 0 (P0). All animal experiments were approved by the Animal Care Committee of the Institute of Medical Science, University of Tokyo and were conducted in accordance with the Association of Research in Vision and Ophthalmology (ARVO) statement for the use of animals in ophthalmic and vision research.

Genotyping and Reverse Transcription and Quantitative PCR (RT-qPCR)

Genomic polymerase chain reaction (PCR) was performed using KOD FX Neo reagents (Toyobo, Japan). The sequences of primers used are followed: *Cre*: 5′-tcgatgcaacgagtgatgag-3′, 5′-ttcggctatacgtaacaggg-3′; *Jmjd3*: 5′-ctgtctgtgatttttgccc cagc-3′, 5′-agggccaatgttgatgttgactg-3′; *Utx*: 5′-gccacatattt ccctagaacacccaggcag-3′, 5′-agctaagctgcttccagggttctcattagc-3′. Total RNA was extracted using Sepasol RNA I Super G (Nacalai tesque, Japan), cDNA was synthesized by ReverTra Ace (Toyobo), and qPCR was performed using Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) and LightCycler 96 (Roche, Basel, Switzerland). The expression level of *Gapdh* and *Sdha* was used for the normalization, and the relative expression level of genes of interest was calculated as previously described.[17] The sequences of primers used are followed; *Gapdh*: 5′-ttagaagcaaacctgtccagcttc-3′, 5′-cataccaggaaatgagcttgac-3′; *Sdha*: 5′-gtgtgaagtagggcaggtcc-3′, 5′-acaaggcactg gctcgatac-3′; *Jmjd3*: 5′- ccatcgctaaatacgcacagtac-3′, 5′-ggccaatgttgatgttgactgag-3′; *Foxn4*: 5′-accactgctctccacaggaataca-3′, 5′-cccatttgccatctgctgcagtc-3′; *Rorb1*: 5′-ggctgggagcttcatgacta-3′, 5′-aatttgtgctcgcatgatgt-3′; *Ptf1a*: 5′-agcctaagcgagaaggcagcaaca-3′, 5′-tggcgagagggtttggctgagaag-3′; *Tfap2a*: 5′-cgttaccctcctcacgtcac-3′, 5′-tgggatcggaatgttgtcgg-3′; *Tfap2b*: 5′-agcggcatgaacctattgga-3′, 5′-aaatacctcgccggtgttga-3′; *Lbx1*: 5′-tagtcctgagcccagcttgc-3′, 5′-ccaccattgaccgacagaga-3′; *Prox1*: 5′-agcctaagcgagaaggcagcaaca-3′, 5′-tggcgagag ggtttggctgagaag-3′; *Atoh7*: 5′-ggcgctcagctacatcatcg-3′, 5′-cccatagggctcagggtctac-3′; *Eomes*: 5′-tccgggacaactacgattcat-3′, 5′-cgggaagaagttttgaacgc-3′; *Nrl*: 5′-cctctataaggcccgctgtg-3′, 5′-gaagaggtgtgtgtggtcgt-3′.

Retinal Explant and Electroporation

Retinal explants culture and electroporation were carried out as described previously.[17] Mouse retinas at E17 were electroporated with pCAG-EGFP and pCAG-KS or pCAG-Jmjd3 plasmids and cultured on a cell culture insert (Millicell, Millipore) for 10 days. Retinal explants were fixed with 4% (wt/vol) paraformaldehyde (PFA) at room temperature for 10 minutes. Frozen sectioned were prepared and immunostained as described below.

Immunostaining

Immunostaining of frozen sectioned retina was performed as previously described.[14] Briefly, eyes were enucleated and fixed with 4% (wt/vol) PFA at room temperature for 10 minutes at E15, E17, and P3, for 15 minutes at P8, or for 30 minutes at P15. After sucrose replacement eyes were embedded in frozen section compound (Leica, Wetzlar, Germany) and sectioned (10 µm or 14 µm) using a cryostat (CM3050S, Leica). Primary antibodies used are followed; rabbit immunoglobulin G (IgG) anti-JMJD3 (ab38113; Abcam, Cambridge, MA, USA), -active caspase-3 (G748A; Promega, Madison, WI, USA), -RBPMS (ab194213; Abcam), -EOMES (ab23345; Abcam), -RXRG (sc-555; Santa Cruz Biotechnology, Dallas, TX, USA), -PH3 (06-570; Millipore, Burlington, MA, USA), mouse IgG anti-TFAP2A (3B5; DSHB, Des Moines, IA, USA), -LHX1 (4F2; DSHB), -Ki67 (550609; BD Bioscience, Franklin Lakes, NJ, USA), -ISL1 (39.4D5; DSHB), and goat IgG anti-BRN3B (sc-6026; Santa Cruz Biotechnology). Appropriate secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (ThermoFisher Scientific, Waltham, MA, USA) were used and analyzed using Axio Imager 2 (Zeiss, Oberkochen, Germany).

Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) was done as previously described.[18] Briefly, mouse whole retinas at P3 were isolated and incubated with 0.25% (wt/vol) trypsin in PBS for 15 minutes at 37°C. Retinas were resuspended with 2% (wt/vol) BSA in PBS and incubated with anti-Cd73 antibody (BD, 561545) and anti-Thy1.2 antibody (BioLegend, 105307) for 30 minutes on ice. Cells were stained with propidium iodide to exclude dead cells. Thy1.2-single-positive and Cd73-single-positive fractions were collected using FACSAria II (BD).

Investigative Ophthalmology & Visual Science

Chromatin Immunoprecipitation and Quantitative Polymerase Chain Reaction

Chromatin immunoprecipitation and quantitative polymerase chain reaction was followed as previously described.[17] Retinas were cross-linked with formalin and sonicated using Sonifier 250A (Branson, Danbury, CT, USA). Rabbit IgG anti-JMJD3 (Abcam, ab38113), -H3K27me3 (39155; Active Motif, Carlsbad, CA, USA) antibody and control rabbit IgG (2729S; Cell Signaling Technology, Danvers, MA, USA) were bound to DynaBeads Protein G (10004D; Life Technologies, Carlsbad, CA, USA) and incubated with sonicated chromatin. Eluate was treated with RNase A (Nacalai, San Diego, CA, USA) and Proteinase K (Fujifilm Wako Pure Chemical Corporation, Wako, Japan). DNA was purified using QIAquick PCR purification kit (Qiagen). The qPCR was done using Thunderbird SYBR qPCR Mix (Toyobo) and LightCycler 96 (Roche). The sequences of primers used are followed; *Rorb1*: 5′-cgcgtataagcctcttctgc-3′, 5′-actgttcagttgcagagacg-3′; *Foxn4*: 5′-tgccctgtaactgaccctta-3′, 5′-atttccctctctggagctgt-3′; *Ptf1a*: 5′-gcgcctgtggatgtagttataag-3′, 5′-tggcactg gagagggatagtac-3′; *Tfap2a*: 5′-aggaagagttgttgggcttg-3′, 5′-tcttctagccgctaacttgctc-3′; *Tfap2b*: 5′-t agtggctgcattgattcgc-3′, 5′-tgggtcacagaatccacagac-3′; *Lhx1*: 5′-aacggacgtcttaccaaacc-3′, 5′-accgcgtttagaggatcagtg-3′; *Eomes*: 5′-caggcgacccgatccaatta-3′, 5′-ccgcttggaaacttgtgagc-3′; *Lhx2*: 5′-actctgctcctcctcctttttc-3′, 5′-agccactgggagaattgaagag-3′; *Atoh7*: 5′-ggtgtgttagttagcgttggac-3′, 5′-acgcacagcaaaatcctgac-3′.

Results

Jmjd3 is Expressed in a Portion of Early-Born Retinal Cells at an Early Embryonic Stage

Previously, we investigated the expression of *Jmjd3* during retinal development by quantitative reverse transcription PCR (RT-qPCR). We found that *Jmjd3* showed enhanced expression in postnatal stages, compared with embryonic stages. Immunostaining revealed that JMJD3 was primarily expressed in the INL and GCL.[17] To further examine the expression of JMJD3 at embryonic stages, mouse retinas at E15 were co-immunostained for JMJD3 and markers of early-born retinal cells. We quantified the proportions of marker and JMJD3 double-positive cells among marker-positive cells; we found that 30% to 50% of TFAP2A-positive ACs, LHX1-positive HCs, and BRN3B-positive RGCs were double-positive (Figs. 1A–C, 1E). We also found that approximately 10% of Ki67-positive RPCs expressed JMJD3 (Figs. 1D, 1E). To evaluate the anti-JMJD3 antibody, a mouse retina at E17 was electroporated with a Jmjd3-overexpressing plasmid and an EGFP-overexpressing plasmid, then immunostained using an anti-JMJD3 antibody. We found that most of EGFP-expressing cells exhibited strong JMJD3 signals, indicating that the antibody recognized JMJD3 (Supplementary Fig. S1).

Jmjd3-cKO in the Retina Resulted in Reductions in the Numbers of ACs and HCs and a Temporal Enhancement of the Number of RGCs

To examine the role of *Jmjd3* in the development of early-born retinal cells, we generated a retina-specific conditional knockout of *Jmjd3* mouse line. We crossed a *Jmjd3*-floxed mouse with a *Dkk3*-Cre mouse and generated a

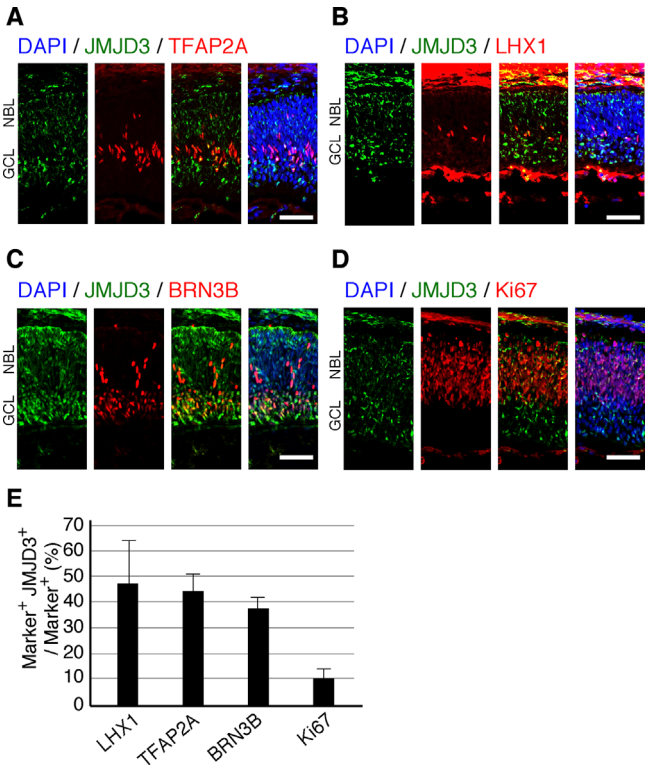

Figure 1. The expression of JMJD3 in the mouse retina at an embryonic stage. Immunostaining of JMJD3 in the mouse retina at E15. Retinas were harvested, frozen-sectioned and co-immunostained with JMJD3 and TFAP2A (**A**), LHX1 (**B**), BRN3B (**C**), or Ki67 (**D**). Cell nuclei were stained with DAPI. The proportions of marker and JMJD3 double-positive cells were quantified and the averages of three independent retinas are shown with standard deviations (**E**). *Scale bar*: 50 µm.

Dkk3-Cre; *Jmjd3*$^{fl/fl}$ (Jmjd3-cKO) mouse. Retinas from *Jmjd3*$^{fl/fl}$ or *Jmjd3*$^{fl/+}$ mice were used as controls. In the *Jmjd3*-floxed mouse, exons 17–19, which encode a portion of the catalytic JmjC domain, are flanked by loxP sites (Fig. 2A). The *Dkk3*-Cre mice express the Cre gene in RPCs beginning at E10.5.[19] RT-qPCR confirmed that the expression of transcripts containing the floxed exons was abolished in the Jmjd3-cKO retina (Fig. 2B). Because exons 17–19 encode 452 of the 721 nucleotides of the JmjC domain, which contains an essential amino acid residue (1388 His) for enzymatic activity of JMJD3,[21] the data indicate that our observation depends on the loss of enzymatic activity of JMJD3. Although the anti-JMJD3 antibody detected a truncated JMJD3 protein in the Jmjd3-cKO retina (Supplementary Fig. S2A), the number of ISL1-positive BCs was significantly reduced in the Jmjd3-cKO retina (Supplementary Fig. S2A, B); this was consistent with the phenotype we observed in the shJmjd3-expressing explant retina.[17] We then investigated the development of early-born retinal subtypes in the Jmjd3-cKO retina by immunostaining analysis of frozen sections of retinas. At E15, TFAP2A-positive ACs were localized in the inner side of the neuroblastic layer (NBL) in the control retina, whereas TFAP2A-positive cells were largely reduced in the Jmjd3-cKO retina (Fig. 2C). At postnatal day 3 (P3), when the production of ACs is completed in the normal mouse retina, TFAP2A-positive ACs were observed in the inner NBL, and the GCL in the control retina. In the

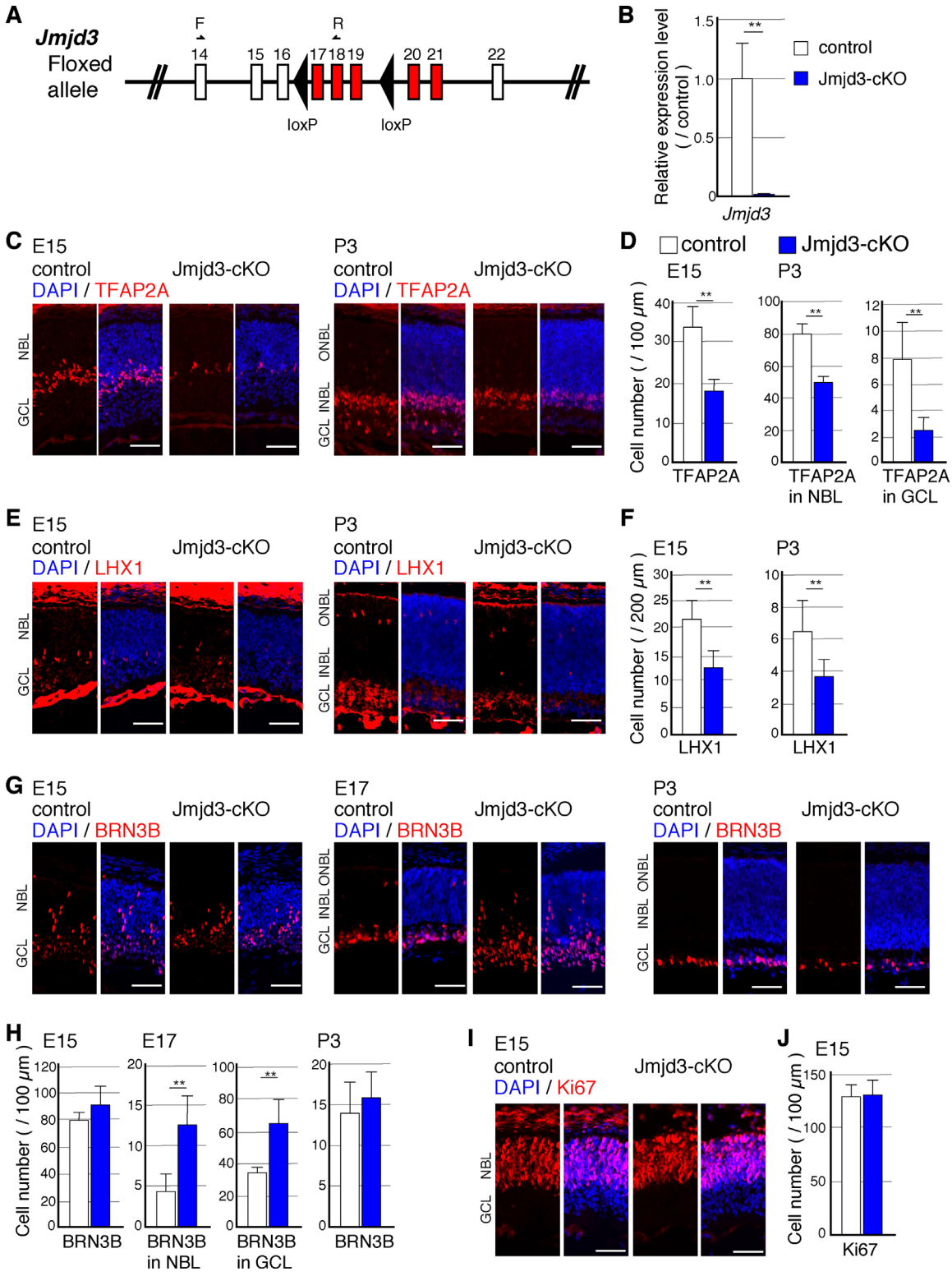

Figure 2. The development of AC, HC, and RGC in the Jmjd3-cKO mouse retina. (**A**) Predicted *Jmjd3* floxed allele. Exons 17–19 of the *Jmjd3* gene were flanked by *loxP* sites. Exons in red encode the Jumonji C domain. Two arrows indicate the position of primers used in *B*. (**B**) The expression level of *Jmjd3* in the Jmjd3-cKO at E15 was examined by RT-qPCR. The averages of three independent mouse retinas with standard deviations are shown. (**C–J**) Immunostaining of the Jmjd3-cKO retina at E15, E17, or P3 with TFAP2A (**C**), LHX1 (**E**), BRN3B (**G**), or Ki67 (**I**). Cell nuclei were stained with DAPI. The three independent mouse retinas were analyzed, and the averages of each marker positive cells with standard deviations are shown in *D*, *F*, *H,* and *J*. INBL, inner neuroblastic layer; ONBL, outer neuroblastic layer. $^{**}P < 0.01$ was calculated by Student's *t* test. *Scale bar*: 50 μm.

Investigative Ophthalmology & Visual Science

Jmjd3-cKO retina, TFAP2A-positive cells were significantly reduced in both the inner NBL and GCL (Figs. 2C, 2D). LHX1-positive HCs were localized on the inner side of the NBL at E15 and in the outer NBL at P3 in the control retina (Fig. 2E). In the Jmjd3-cKO retina, the numbers of LHX1-positive cells were reduced by approximately 50% at both E15 and P3 (Figs. 2E, 2F). To investigate whether Jmjd3 plays pivotal roles in the production or the maintenance of ACs and HCs during early retinal development, we examined apoptotic cell death by staining with an active caspase-3 antibody. We did not find differences in the numbers of active caspase-3-positive cells at E15, E17, and P3 (Supplementary Fig. S2C, D), suggesting that *Jmjd3* is required for production, but not maintenance, of ACs and HCs.

Next, we examined the development of RGCs in the Jmjd3-cKO retina by immunostaining. At E15, BRN3B-positive RGCs were detected in the NBL and GCL in both the control and Jmjd3-cKO retinas, and the numbers of BRN3B-positive cells were comparable (Figs. 2G, 2H). Notably, at E17, when most of the BRN3B-positive RGCs in the control retina were localized to the GCL, BRN3B-positive cells remained in the NBL of the Jmjd3-cKO retina; the numbers of BRN3B-positive cells were elevated in the GCL and NBL in the Jmjd3-cKO retina (Figs. 2G, 2H). At P3, when the production of RGCs is complete in normal retinal development, the numbers of BRN3B-positive cells in the control and Jmjd3-cKO retinas were not significantly different (Figs. 2G, 2H). Although the numbers of ACs and HCs were reduced in the Jmjd3-cKO retina, the number of RGCs was elevated. Because we did not find differences in the numbers of active caspase-3– and phosphohistone H3 (PH3)–positive cells at E17 (Supplementary Figs. S2C, S2D, S2G, S2H), the results suggested that altered apoptosis or proliferation did not cause the elevated RGCs in the Jmjd3-cKO retina.

Cones are other early-born retinal cells, which express RXRG from an early developmental stage. RXRG-positive cones were observed in the outer NBL of the Jmjd3-cKO retina at E15 and P3 (Supplementary Fig. S2E). The numbers of RXRG-positive cells were comparable between Jmjd3-cKO and control retinas (Supplementary Fig. S2F), indicating a dispensable role of Jmjd3 in cone development. Although some RPCs expressed JMJD3 (Figs. 1D, 1E), we did not find differences in the numbers of Ki67-positive and PH3-positive cells in the Jmjd3-cKO retina (Figs. 2I, 2J, S2G, S2H). This finding suggests that the reductions in ACs and HCs were not caused by the depletion of RPCs.

Aberrant Expression of Transcription Factors Involved in the Development of ACs, HCs, and RGCs in the Jmjd3-cKO Retina

Studies have revealed the genetic cascade of the transcription factors that regulate the development of ACs, HCs, and RGCs (Fig. 3A).[22–25] *Rorb1*, an isoform of *Rorb*, and *Foxn4* are expressed in some RPCs; these factors promote the commitment to ACs and HCs.[24,25] *Ptf1a* is a downstream target gene of *Rorb1* and *Foxn4*, which contributes to the development of ACs and HCs in post-mitotic cells.[22] *Tfap2a* and *Tfap2b* are downstream genes of *Ptf1*a that partly mediate the role of *Ptf1a* in AC development.[23] *Lhx1* is involved in HC development and is presumably downstream of *Tfap2a* and *Tfap2b*.[23,26]

We investigated the expression levels of these transcription factors in the Jmjd3-cKO retina at E15 and found that *Ptf1a* and *Tfap2a* expression levels were significantly reduced in the Jmjd3-cKO retina (Fig. 3B), whereas expression levels of *Foxn4* and *Rorb1* (both expressed in RPCs) were not. Although the differences were not statistically significant, the expression levels of *Tfap2b*, *Prox1*, and *Lhx1* were slightly reduced in the Jmjd3-cKO retina. However, we cannot exclude the possibility that an unknown critical gene for HC and AC genesis was suppressed in the Jmjd3-cKO retina. Importantly, contrasting AC and HC development, *Ptf1a* inhibits the production of RGCs by repressing the expression of *Atoh7*,[22,27,28] which is critical for RGC development.[29] Therefore reduced *Ptf1a* expression might lead to elevated numbers of RGCs by enhancement of *Atoh7* expression. The expression of *Atoh7* was slightly enhanced in the Jmjd3-cKO retina at E15, but the difference was not statistically significant (Fig. 3B). However, enhanced *Atoh7* expression was observed at E17 (Fig. 3B), suggesting that *Jmjd3* negatively regulates the expression of *Atoh7* through regulation of *Ptf1a* expression.

Jmjd3 Binds the Promoter Regions of Transcription Factors Involved in the Development of ACs, HCs, and RGCs

We then assessed the levels of H3K27me3 around the promoter regions of these transcription factors in whole retinas at E14.5 and E17.5, using a publicly accessible database (https://pecan.stjude.cloud/proteinpaint/study/retina2017).[30] We found a relatively high H3K27me3 signal in the promoter regions of several transcription factors, including *Ptf1a*, *Tfap2a*, *Tfap2b*, and *Lhx1* (Supplementary Fig. S3). Considering the small population of postmitotic cells committed to ACs and HCs, we presume that Jmjd3-mediated derepression via H3K27me3 demethylase activity is required for the expression of these transcription factors.

To examine whether *Jmjd3* directly regulates the expression of the above transcription factors by binding their promoter regions, we performed ChIP-qPCR analysis using a normal whole retina at E15. The results indicated that JMJD3 bound the promoter regions of *Ptf1a*, *Tfap2a*, *Tfap2b*, and *Lhx1*. In contrast, we did not observe JMJD3 binding in the promoter regions of *Rorb*1, *Foxn4*, and *Lhx2*, which are active in the RPCs (Fig. 3C). Furthermore, JMJD3 did not bind to *Atoh7* (Fig. 3C), supporting our hypothesis that JMJD3 indirectly regulates the *Atoh7* expression by controlling *Ptf1a* expression. These results that the Jmjd3 contributes to the development of ACs, HCs, and RGCs by regulating the expression levels of various transcription factors, including *Ptf1a*, *Tfap2a*, *Tfap2b*, and *Lhx1*.

Jmjd3 Contributes to the Maintenance of RGCs, Presumably by Regulation of Eomes Expression

The numbers of RGCs at P3 were comparable between the Jmjd3-cKO and control retinas (Figs. 2G, 2H). At later stages, numbers of BRN3B-positive RGCs were reduced in the Jmjd3-cKO retina (Figs. 4A, 4B). We confirmed the reduced number of RGCs by using another protein specifically expressed in RGCs, the pan-RGC marker RBPMS. We found fewer RBPMS-positive RGCs at P8 and P15 (Figs. 4A, 4B), indicating a role for *Jmjd3* in maintenance of RGCs during late retinal development. *Eomes* (*Tbr2*), a T-box

Investigative Ophthalmology & Visual Science

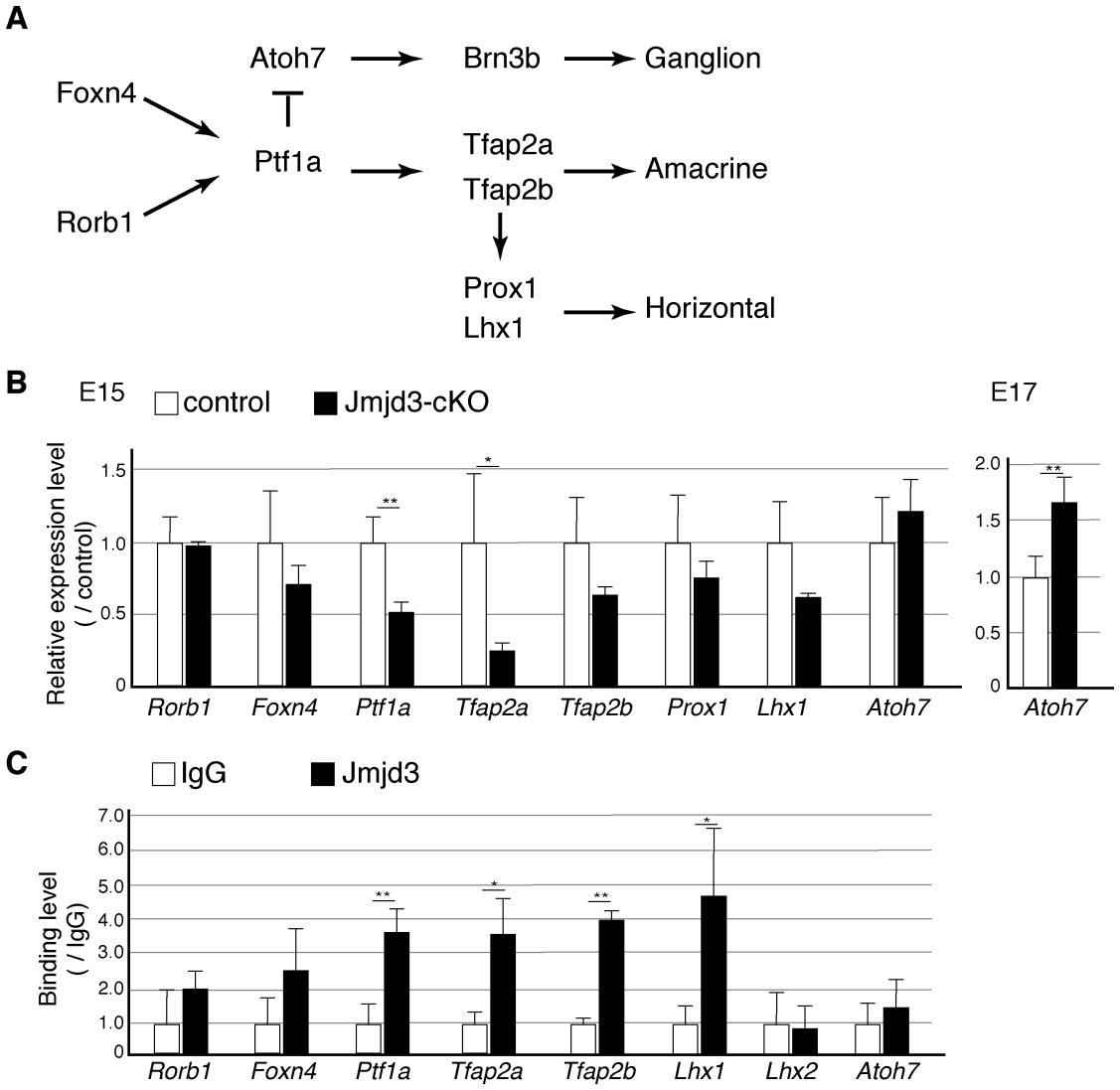

Figure 3. The molecular mechanisms by which *Jmjd3* contributes to the development of AC, HC, and RGC. (**A**) The cascade of transcription factors involved in the development of AC, HC, and RGC. *Foxn4* and *Rorb1* are expressed in some RPCs and induce the commitment to AC and HC through regulating the expression of downstream transcription factors, including *Ptf1a*, *Tfap2a*, *Tfap2b* and *Lhx1* in postmitotic cells. *Ptf1a* inhibits the production of RGCs by repressing *Atoh7* expression. (**B**) The expression of the transcription factors related to the development of AC, HC, and RGC in the embryonic Jmjd3-cKO retina. The mRNA expression levels of transcription factors shown in *A* were analyzed by RT-qPCR. The relative expression levels of each gene in the Jmjd3-cKO retina at E15 and E17 were calculated. The averages of three independent retinas are shown with standard deviations. (**C**) The binding of JMJD3 in the promoter region of the transcription factors. ChIP-qPCR analysis using control IgG or JMJD3 antibody with mouse whole retinas at E15 was performed. The percentage of input of JMJD3 relative to that of control IgG was calculated as binding level. The averages of three independent retinas are shown with standard deviations. $^{**}P < 0.01$, $^{*}P < 0.05$ was calculated by Student's *t* test.

transcription factor, is directly regulated by *Brn3b* and is important for RGC development.[31] *Eomes* is expressed in intrinsically photosensitive RGCs and contributes to the formation and maintenance of those cells.[32] *Eomes* also regulates endodermal differentiation, and Jmjd3-mediated derepression of *Eomes* expression is critical.[33] Immunostaining revealed reduced numbers of EOMES-positive RGCs in the Jmjd3-cKO retina at P8 and P15 (Figs. 4A, 4B). The reduction in EOMES-positive cells was more drastic than the reduction of RBPMS-positive cells. Subsequently, *Eomes* expression was examined by RT-qPCR. *Eomes* expression was lower in the Jmjd3-cKO retina than in the control retina at P3 (Fig. 4C), although the numbers of RGCs were comparable between the Jmjd3-cKO and control retinas (Supplementary Fig. S2I, S2J). We also examined the H3K27me3 level in the promoter region of *Eomes* in the Jmjd3-cKO retina by ChIP-qPCR; the results showed a significantly elevated H3K27me3 level in the Jmjd3-cKO retina at P15 (Fig. 4D). To examine the levels of H3K27me3 and JMJD3 binding in the promoter region of *Eomes* in RGCs, RGCs and rods were separated by flow cytometry using Thy1.2 and CD73, which are markers for RGCs and rods, respectively (Supplementary Fig. S4A, S4B).[34–36] H3K27me3 and JMJD3 binding in the promoter region of *Eomes* were examined by ChIP-qPCR (Supplementary Fig. S4A). First, we investigated the purity of the fractionated cells by examining the expression levels of *Eomes* and *Nrl* by RT-qPCR; RGCs and rods were enriched more than 30-fold (Supplementary Fig. S4C). ChIP-qPCR revealed that

Investigative Ophthalmology & Visual Science

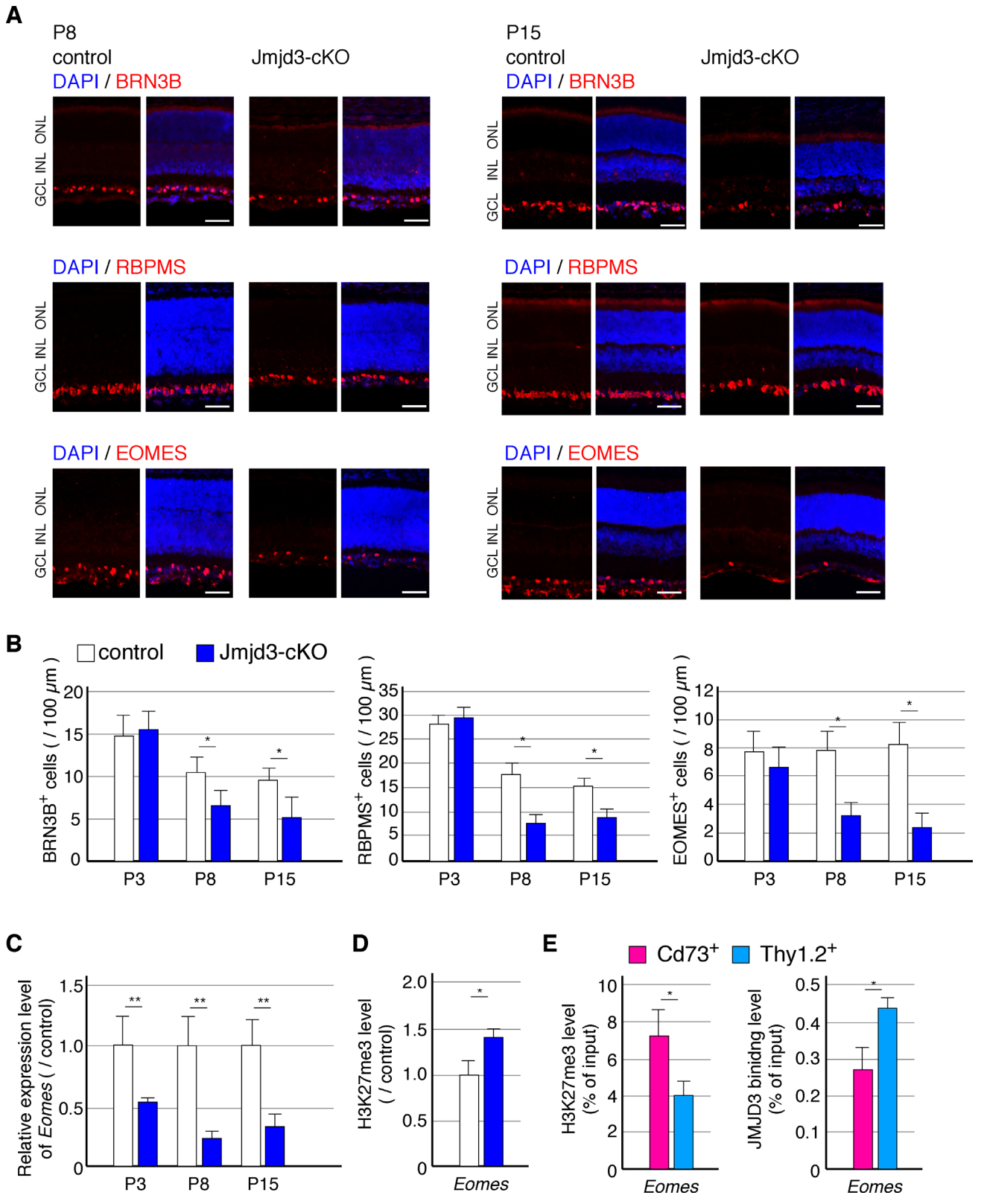

Figure 4. The contribution of *Jmjd3* to the maintenance of RGCs presumably by regulation of *Eomes* expression. (**A, B**) The Jmjd3-cKO retinas at postnatal stages were harvested, cryosectioned and immunostained with BRN3B, RBPMS, or EOMES. Cell nuclei were stained with DAPI. The averages of marker positive cells in three independent retinas are shown with standard deviations. (**C**) The mRNA expression level of *Eomes* in the postnatal Jmjd3-cKO retinas. RT-qPCR analysis using the whole retinas at indicated stages was performed. The averages of three independent retinas are shown with standard deviations. (**D**) The H3K27me3 level in the promoter region of *Eomes* in the Jmjd3-cKO retina at P15. Relative H3K27me3 level normalized by control was calculated. The averages of three independent retinas are shown with standard deviations. (**E**) The H3K27me3 level and JMJD3 binding in the promoter region of *Eomes* in RGCs- or rod-enriched fraction. Cd73-epxressing rods and Thy1.2-expressing RGCs in retinas at P3 were purified by FACS, and ChIP-qPCR analysis using H3K27me3 or JMJD3

Investigative Ophthalmology & Visual Science

antibody was performed. The percentage of input was calculated. The averages of three independent samples are shown with standard deviations. ONL, outer nuclear layer. $^{**}P < 0.01$, $^{*}P < 0.05$ was calculated by Student's *t* test. *Scale bar*: 50 µm.

the H3K27me3 level in the *Eomes* promoter region was lower in the RGC-enriched fraction, which expresses *Eomes*, than in the rod-enriched fraction, which does not express *Eomes* (Fig. 4E). In addition, JMJD3 was found to bind the *Eomes* promoter region in RGC-enriched fraction (Fig. 4E), supporting our hypothesis that Jmjd3 derepresses *Eomes* expression by demethylation of H3K27me3 in the promoter region of *Eomes* in postnatal RGCs, thereby contributing to the maintenance of RGCs.

RGCs and ACs were Not Abolished in the Jmjd3 and Utx Conditional Double Knockout (Jmjd3/Utx-cDKO) Mouse Retina

Although the numbers of RGCs and ACs were reduced, we did not observe complete loss of RGCs and ACs in the Jmjd3-cKO retina. *Utx* is another H3K27me3-specific demethylase that is expressed in the INL and GCL during retinal development.[37] We found that the numbers of BRN3B-positive RGCs and TFAP2A-positive ACs were not reduced in the Utx-cKO retina (Supplementary Figs. S5A–D). Because Jmjd3-cKO did not result in a complete loss of RGCs, we suspected compensatory roles for Utx in Jmjd3-cKO; we investigated whether *Jmjd3* and *Utx* cooperatively contribute to the maintenance of RGCs or ACs using double knockout mice of *Jmjd3* and *Utx*. To exclude the presence of Uty, which is localized in the Y-chromosome and may affect the results, we used only female *Dkk3*-Cre; $Jmjd3^{fl/fl}$; $Utx^{fl/fl}$ (Jmjd3/Utx-cDKO) mice. Although the Jmjd3/Utx-cDKO mouse retina showed reductions in BRN3B-positive RGCs and TFAP2A-positive ACs at P15, the phenotype was comparable with that of the Jmjd3-cKO retina (Supplementary Figs. S5A–D), suggesting the presence of a pathway independent of *Jmjd3* and *Utx*.

Discussion

We previously demonstrated the role of Jmjd3 during development of BC subsets by using in vitro retinal organ culture.[17] This work is an extension of our in vitro study; we found that proper differentiation of early-born retinal lineages (such as ACs and HCs) was impaired in the Jmjd3-cKO retina. Notably, the number of developing RGCs was elevated, and we found that that Ptf1a may be a key factor underlying the difference in phenotypes between RGCs and ACs/HCs. We observed scattered cells expressing BRN3B in the Jmjd3-cKO retina, suggesting that migration of RGCs to the GCL was delayed or that the production period of RGCs was extended in the Jmjd3-cKO retina. Alternatively, ectopic expression of derepressed BRN3B in cells other than the RGC lineage is plausible. Furthermore, the maintenance of RGCs is also dependent on Jmjd3. When we began to investigate H3K27 methylation in the retina, we considered the possibility that Jmjd3 might be essential for all retinal cells, regardless of cell type; however, our in vitro and in vivo analyses clearly showed that the role of *Jmjd3* is relatively restricted. Surprisingly, double knockout of *Jmjd3* and *Utx* in the retina did not show synergistic or additive effects.

T-box signaling is critical for endoderm differentiation, and TBX3 binds JMJD3 at the enhancer region of *Eomes*, leading to its expression.[33] EOMES itself interacts with JMJD3 and enhances its own expression. We observed strong enhancement of *Eomes* expression, reduced H3K27me3 level, and JMJD3 binding at the *Eomes* locus in RGCs. In addition, the H3K27me3 level of *Eomes* was enhanced in the Jmjd3-cKO retina. Thus EOMES may recruit JMJD3 to the promoter region of *Eomes* and maintain derepression of *Eomes* transcripts in postnatal RGCs. Using single-cell RNA-Seq (scRNA-Seq) data concerning the developing mouse retina,[38] we found that 30% of *Eomes*-expressing RGCs expressed *Jmjd3*, suggesting the coexpression of JMJD3 and EOMES in RGCs. The *Tbx3* transcript is also expressed in the retina (data not shown), and involvement of *Tbx2* or other members is plausible.

We showed that some, but not all, ACs and HCs express JMJD3 at an embryonic stage; this was supported by scRNA-Seq findings that 15%–20% of ACs or HCs expressed *Jmjd3*. Regarding the mechanism of AC and HC differentiation, the transcriptional network has been studied; Ptf1a is a key transcription factor, and a study showed that RORB1 and FOXN4 cooperatively enhance the expression of *Ptf1a*.[25] FOXN4 is expressed only in some mitotic RPCs, whereas RORB1 is expressed in mitotic RPCs and postmitotic AC and HC precursors.[24,25] Our immunostaining results showed that JMJD3 is expressed in some postmitotic cells, including ACs and HCs, whereas JMJD3 is expressed in a few RPCs. Thus RORB1 and JMJD3 may form an enhancer-promoter loop that activates *Ptf1a* expression.

Because *Jmjd3* has no DNA-binding domain, interacting molecules specific to certain retinal cell types are presumed to lead JMJD3 to target loci. During early endoderm differentiation, SMAD2 and SMAD3 have been suggested to recruit JMJD3 to target promoter regions and stimulate endoderm commitment.[39] Both Smad2 and Smad3 are continually expressed in developing and developed retina (data not shown); we found that forced expression of constitutively active BMP, an upstream regulator of SMADs, led to enhanced BP and Müller glia genesis.[40] Another group showed that BMP signaling is required for Müller glia differentiation.[41] Although we did not observe perturbation of the Müller glia lineage, SMADs may participate in BP differentiation, in collaboration with *Jmjd3*.

Recently, we found that another H3K27me3 demethylase, *Utx,* is also required for the development of rod-ON-BCs in a manner similar to that of *Jmjd3*.[37] Because the gross expression patterns of *Jmjd3* and *Utx* are different, we surmised that *Utx* might divide demethylation in retinal development and regulate retinal cells other than BCs. Therefore one important question that remains to be addressed is whether other lineages of retinal cells (such as photoreceptors or Müller glia) require demethylation of H3K27me3 for differentiation. We hypothesize that another H3K27me3-specific demethylase exists, but thus far, no supporting evidence has been obtained. Although a redundant phenotype was observed in loss of function of *Utx* or *Jmjd3*, Jmjd3/Utx-cDKO mice did not show complete loss of PKCα-positive rod-ON-BCs.[37] In this study, we observed reductions in the numbers of ACs and RGCs in the Jmjd3-cKO retina. Although both demethylases are strongly expressed in postnatal RGCs and ACs, the Jmjd3/Utx-cDKO retina showed a reduction,

Investigative Ophthalmology & Visual Science

but not a complete loss, of RGCs and ACs; currently, we do not know the underlying mechanism. Recent studies have revealed the demethylation-independent functions of Jmjd3 and Utx, such as acetylation of H3K27 and trimethylation of H3K4.[42] We observed expression of truncated JMJD3 in Jmjd3-cKO retinas; thus truncated JMJD3 might retain some activity, leading to incomplete loss of BCs, RGCs, or ACs in the double knockout retina. However, we surmise that the roles of *Jmjd3* and *Utx* may not be in deciding cell fate; *Jmjd3* and *Utx* may be involved in precise regulation of cell numbers during differentiation and maintenance of differentiated cell lineages. This notion is consistent with the involvement of other positive marks of histone modification in retinal cell fate.

Acknowledgments

The authors thank Asano Tsuhako and Miho Nagoya for their excellent technical assistance.

Supported by JSPS KAKENHI (Grant Number 17H05760 to S. Watanabe, 18K16949 to T. Iwagawa).

Disclosure: **T. Iwagawa,** None; **H. Honda,** None; **S. Watanabe,** None

References

1. Cepko CL, Austin CP, Yang X, Alexiades M, Ezzeddine D. Cell fate determination in the vertebrate retina. *Proc Natl Acad Sci USA*. 1996;93:589–595.
2. Young RW. Cell differentiation in the retina of the mouse. (Article, 1985) [WorldCat.org]. *Anat Rec*. 1985;212:199–205.
3. Brzezinski JA, Reh TA. Photoreceptor cell fate specification in vertebrates. *Development*. 2015;142:3263–3273.
4. Cepko C. Intrinsically different retinal progenitor cells produce specific types of progeny. *Nat Rev Neurosci*. 2014;15:615–627.
5. Swaroop A, Kim D, Forrest D. Transcriptional regulation of photoreceptor development and homeostasis in the mammalian retina. *Nat Rev Neurosci*. 2010;11:563–576.
6. Xiang M. Intrinsic control of mammalian retinogenesis. *Cell Mol Life Sci*. 2013;70:2519–2532.
7. Corso-Díaz X, Jaeger C, Chaitankar V, Swaroop A. Epigenetic control of gene regulation during development and disease: A view from the retina. *Prog Retin Eye Res*. 2018;65(February):1–27.
8. Zelinger L, Swaroop A. RNA Biology in Retinal Development and Disease. *Trends Genet*. 2018;34:341–351.
9. Iwagawa T, Watanabe S. Molecular mechanisms of H3K27me3 and H3K4me3 in retinal development. *Neurosci Res*. 2019;138:43–48.
10. Schuettengruber B, Martinez AM, Iovino N, Cavalli G. Trithorax group proteins: Switching genes on and keeping them active. *Nat Rev Mol Cell Biol*. 2011;12:799–814.
11. Kooistra SM, Helin K. Molecular mechanisms and potential functions of histone demethylases. *Nat Rev Mol Cell Biol*. 2012;13:297–311.
12. Margueron R, Reinberg D. The Polycomb complex PRC2 and its mark in life. *Nature*. 2011;469:343–349.
13. Aldiri I, Moore KB, Hutcheson DA, Zhang J, Vetter ML. Polycomb repressive complex PRC2 regulates Xenopus retina development downstream of Wnt/β-catenin signaling. *Dev*. 2013;140:2867–2878.
14. Iida A, Iwagawa T, Baba Y, et al. Roles of histone H3K27 trimethylase Ezh2 in retinal proliferation and differentiation. *Dev Neurobiol*. 2015;75:947–960.
15. Zhang J, Taylor RJ, La Torre A, et al. Ezh2 maintains retinal progenitor proliferation, transcriptional integrity, and the timing of late differentiation. *Dev Biol*. 2015;403:128–138.
16. Yan N, Cheng L, Cho K, et al. Postnatal onset of retinal degeneration by loss of embryonic Ezh2 repression of Six1. *Sci Rep*. 2016;6(September:1–15.
17. Iida A, Iwagawa T, Kuribayashi H, et al. Histone demethylase Jmjd3 is required for the development of subsets of retinal bipolar cells. *Proc Natl Acad Sci USA*. 2014;111:3751–3756.
18. Ueno K, Iwagawa T, Kuribayashi H, et al. Transition of differential histone H3 methylation in photoreceptors and other retinal cells during retinal differentiation. *Sci Rep*. 2016;6:29264.
19. Sato S, Inoue T, Terada K, et al. Dkk3-Cre BAC transgenic mouse line: a tool for highly efficient gene deletion in retinal progenitor cells. *genesis*. 2007;45:502–507.
20. Umutoni D, Iwagawa T, Baba Y, et al. H3K27me3 demethylase UTX regulates the differentiation of a subset of bipolar cells in the mouse retina. *Genes to Cells*. 2020;25:402–412.
21. De Santa F, Totaro MG, Prosperini E, Notarbartolo S, Testa G, Natoli G. The histone H3 lysine-27 demethylase Jmjd3 links inflammation to inhibition of polycomb-mediated gene silencing. *Cell*. 2007;130:1083–1094.
22. Fujitani Y, Fujitani S, Luo H, et al. Ptf1a determines horizontal and amacrine cell fates during mouse retinal development. *Development*. 2006;133:4439–4450.
23. Jin K, Jiang H, Xiao D, Zou M, Zhu J, Xiang M. Tfap2a and 2b act downstream of Ptf1a to promote amacrine cell differentiation during retinogenesis. *Mol Brain*. 2015;8:1–14.
24. Li S, Mo Z, Yang X, Price SM, Shen MM, Xiang M. Foxn4 controls the genesis of amacrine and horizontal cells by retinal progenitors. *Neuron*. 2004;43:795–807.
25. Liu H, Kim SY, Fu Y, et al. An isoform of retinoid-related orphan receptor β directs differentiation of retinal amacrine and horizontal interneurons. *Nat Commun*. 2013;4(May):1813.
26. Poché RA, Kin MK, Raven MA, Furuta Y, Reese BE, Behringer RR. Lim1 is essential for the correct laminar positioning of retinal horizontal cells. *J Neurosci*. 2007;27:14099–14107.
27. Lelièvre EC, Lek M, Boije H, et al. Ptf1a/Rbpj complex inhibits ganglion cell fate and drives the specification of all horizontal cell subtypes in the chick retina. *Dev Biol*. 2011;358:296–308.
28. Nakhai H, Sel S, Favor J, et al. Ptf1a is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina. *Development*. 2007;134:1151–1160.
29. Brown NL, Patel S, Brzezinski J, Glaser T. Math5 is required for retinal ganglion cell and optic nerve formation. *Development*. 2001;128:2497–2508.
30. Aldiri I, Xu B, Wang L, et al. The dynamic epigenetic landscape of the retina during development, reprogramming, and tumorigenesis. *Neuron*. 2017;94:550–568.e10.
31. Mao CA, Kiyama T, Pan P, Furuta Y, Hadjantonakis AK, Klein WH. Eomesodermin, a target gene of Pou4f2, is required for retinal ganglion cell and optic nerve development in the mouse. *Development*. 2008;135:271–280.
32. Mao CA, Li H, Zhang Z, et al. T-box transcription regulator Tbr2 is essential for the formation and maintenance of Opn4/melanopsin-expressing intrinsically photosensitive retinal ganglion cells. *J Neurosci*. 2014;34:13083–13095

Investigative Ophthalmology & Visual Science

33. Kartikasari AE, Zhou JX, Kanji MS, et al. The histone demethylase Jmjd3 sequentially associates with the transcription factors Tbx3 and Eomes to drive endoderm differentiation. *EMBO J*. 2013;32:1393–1408.
34. Koso H, Minami C, Tabata Y, et al. CD73, a novel cell surface antigen that characterizes retinal photoreceptor precursor cells. *Investig Ophthalmol Vis Sci*. 2009;50:5411–5418.
35. Barres BA, Silverstein BE, Corey DR, Chun LLY. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron*. 1988;1:791–803.
36. Hong S, Iizuka Y, Kim CY, Seong GJ. Isolation of primary mouse retinal ganglion cells using immunopanning-magnetic separation. *Mol Vis*. 2012;18(December):2922–2930.
37. Watanabe S, Murakami A. H3K27me3 demethylase UTX regulates the differentiation of a subset of bipolar cells in the mouse retina. *Adv Exp Med Biol*. 2016;854:635–641.
38. Clark BS, Stein-O'Brien GL, Shiau F, et al. Single-cell RNA-Seq analysis of retinal development identifies NFI factors as regulating mitotic exit and late-born cell specification. *Neuron*. 2019;102:1111–1126.
39. Kim SW, Yoon SJ, Chuong E, et al. Chromatin and transcriptional signatures for Nodal signaling during endoderm formation in hESCs. *Dev Biol*. 2011;357:492–504.
40. Kuribayashi H, Baba Y, Watanabe S. BMP signaling participates in late phase differentiation of the retina, partly via upregulation of Hey2. *Dev Neurobiol*. 2014;74:1172–1183.
41. Ueki Y, Wilken MS, Cox KE, Chipman LB, Bermingham-Mcdonogh O, Reh TA. A transient wave of bmp signaling in the retina is necessary for muller glial differentiation. *Dev*. 2015;142:533–543.
42. Arcipowski KM, Martinez CA, Ntziachristos P. Histone demethylases in physiology and cancer: a tale of two enzymes, JMJD3 and UTX. *Curr Opin Genet Dev*. 2016;36:59–67.

Investigative Ophthalmology & Visual Science