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

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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
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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. However, examination of the Eh2-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.

Although our previous study showed that Jmjd3 is expressed from embryonic stages and strongly expressed in the postnatal GCL, 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), were crossed with Jmjd3 floxed and Utx floxed mice. 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 ophthalamic 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′-tcggctgatatgctcgccgagtggag-3′, 5′-ctgctacggagagcgctgttcgcg-3′, 5′-ggcctgaagctggagagacctgac-3′; Jmjd3: 5′-ctgtgctgatgttgctgcgactg-3′, 5′-agccagctgctgtgcgagct-3′, 5′-acaggactgac-3′; Sdhb: 5′-gtggaagatgacgacaggtg-3′, 5′-acaggactgac-3′; Jmjd3: 5′-ccatgctaatagcagcagctac-3′, 5′-ggccattaagttgattgctgag-3′; Foxn4: 5′-acaacctgtctccccagggtagataca-3′, 5′-ccattgctacctgcctgac-3′; Rorb1: 5′-ggcggagatctgacta-3′, 5′-aatggctgcctgacctgt-3′; Ptfla: 5′-agctaagcaggaagagcagcagc-3′, 5′-tggcggagagctctgtgctgag-3′; Tfaq2a: 5′-ctgatcctccctcactcgc-3′, 5′-tggagctcggatgttgccg-3′; Tfaq2b: 5′-agccagcaacctattgga-3′, 5′-aatatcctcgcctgttgga-3′; Lbx1: 5′-tacctgcggccacagctgt-3′, 5′-caccattagcgacgaca-3′; Proxl: 5′-agcttgctaggaagagcagcagc-3′, 5′-tggcggagagttggtcctgag-3′; Atoh7: 5′-gggcgtcagctacatcgc-3′, 5′-cctataggtcaggtctcctg-3′; Eomes: 5′-tcggccggaactacgtgagt-3′, 5′-ggggaagatgtttgaaccgc-3′; Nrl: 5′-cccttaaaacgcgcctgtg-3′, 5′-gaagaggtgtggtctgct-3′.

Retinal Explant and Electroporation

Retinal explants culture and electroporation were carried out as described previously. 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. 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), -RBPM5 (ab194213; Abcam), -EOMES (ab23543; Abcam), -RXRG (sc-555; Santa Cruz Biotechnology, Dallas, TX, USA), -PFl3 (06-570; Millipore, Burlington, MA, USA), mouse IgG anti-TFAP2A (3B5; DSHB, 06-570; Millipore, Burlington, MA, USA), mouse IgG anti-TRAF2 (3B5; DSHB, 06-570; Millipore, Burlington, MA, USA), mouse IgG anti-BAM3 (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 Axios Imager 2 (Zeiss, Oberkochen, Germany).

Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) was done as previously described. 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- and BCs were collected positive and Cd73-single-positive fractions were collected using FACSARia II (BD).
Chromatin Immunoprecipitation and Quantitative Polymerase Chain Reaction

Chromatin immunoprecipitation and quantitative polymerase chain reaction was followed as previously described. 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′-caggcgacccgatccaatta-3′; Foxn4: 5′-tgccctgtggatgtagttataag-3′; Ptf1a: 5′-gcgcctgtggatgtagttataag-3′; Ptfa1a: 5′-gcgcctgtggatgtagttataag-3′; 5′-tggcactg gaggaggtgatagc-3′; Tfap2a: 5′-aggragaggtttggtggctg-3′; 5′-ttcttgagccgctaattgcg-3′; Tfap2b: 5′-agttgctgcttgattgcg-3′; 5′-tggtctcgatgacacacgacag-3′; Lhx1: 5′-aacggaagcttcaaaac-3′; 5′-acgcgtttgaggtcgtg-3′; Eomes: 5′-ccgagcccttgacac-3′; 5′-ccgagctttgactgacagc-3′; Lhx2: 5′-actctgtctctctctctctctctctctctctct-3′; 5′-agccactgacaggtc-3′; Atoh7: 5′-agtttgctgatgagtgatagc-3′; 5′-acgcctgaccaaaatcttgac-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. 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 retina at E17 was electroporated with a Jmjd3-overexpressing plasmid and an EGFP-overexpressing plasmid, and 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 Dkk3-Cre mouse with a Dkk3-Cre mouse and generated a Jmjd3-cKO in the retina at an early 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).

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.
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.
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Jmjd3-cKO retina, TFAP2A-positive cells were significantly reduced in both the inner NBL and GCL (Figs. 2C, 2D). LH1X-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 LH1X-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). Rorb1, an isoform of Rorb, and Foxn4 are expressed in some RPCs; these factors promote the commitment to ACs and HCs. Ptf1a is a downstream target gene of Rorb1 and Foxn4, which contributes to the development of ACs and HCs in post-mitotic cells. Tfap2a and Tfap2b are downstream genes of Ptf1a that partly medi ate the role of Ptf1a in AC development. Lhx1 is involved in HC development and is presumably downstream of Tfap2a and Tfap2b.

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, which is critical for RGC development. 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). 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 Rorb1, 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 (Thb2), a T-box
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. Eomes is expressed in intrinsically photosensitive RGCs and contributes to the formation and maintenance of those cells. Eomes also regulates endodermal differentiation, and Jmjd3-mediated derepression of Eomes expression is critical. 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). 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...
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-expressing rods and Thy1.2-expressing RGCs in retinas at P3 were purified by FACS, and ChIP-qPCR analysis using H3K27me3 or JMJD3.
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 Utx, which is localized in the Y-chromosome and may affect the results, we used only female Dkk3-Cre; Jmjd3<sup>fl/fl</sup>; Utx<sup>fl/fl</sup> (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.37 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 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 ONL, outer nuclear layer. **P < 0.01, *P < 0.05 was calculated by Student’s t test. Scale bar: 50 μm.**
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.

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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, Reubin 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;393:128–138.
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. Umuton 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. Tflp2a and 2b act downstream of Ptf1a to promote amacrine cell differentiation during retinogenesis. Mol Brain. 2015;8:14–1.
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. Poche 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/Bhp1 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 development. Development. 2001;128:497–508.
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. Eomesoderm, 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.
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.