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Highlights
MLL5 is essential for the expression of critical photoreceptor genes
MLL5 depletion reduces H3K4/K79 methylation at photoreceptor gene promoters
MLL5 interacts with CRX via its CD4 domain
Recognition of H3K4me2/3 by MLL5 is a prerequisite for CRX recruitment to chromatin

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Xiaoming Zhang, Bo-Wen Zhang, Lue Xiang, Hui Wu, SUPIT Alva Sahiri Alexander, Peipei Zhou, Melvin Zi-Yu Dai, Xiaoyun Wang, Wenjun Xiong, Yan Zhang, Zi-Bing Jin, and Lih-Wen Deng

SUMMARY

Histone methylation, particularly at the H3K4 position, is thought to contribute to the specification of photoreceptor cell fate; however, the mechanisms linking histone methylation with transcription factor transactivation and photoreceptor gene expression have not yet been determined. Here, we demonstrate that MLL5 is abundantly expressed in the mouse retina. Mll5 deficiency impaired electroretinogram responses, alongside attenuated expression of a number of retina genes. Mechanistic studies revealed that MLL5 interacts with the retina-specific transcription factor, CRX, contributing to its binding to photoreceptor-specific gene promoters. Moreover, depletion of MLL5 impairs H3K4 methylation and H3K79 methylation, which subsequently compromises CRX-CBP assembly and H3 acetylation on photoreceptor promoters. Our data support a scenario in which recognition of H3K4 methylation by MLL5 is required for photoreceptor-specific gene transcription through maintaining a permissive chromatin state and proper CRX-CBP recruitment at promoter sites.

INTRODUCTION

The vertebrate retina comprises six major types of neurons (cone, rod, bipolar, amacrine, horizontal, and ganglion cells) and one type of glial cells (Müller glia) derived from a common population of multipotent progenitor cells that develop in a conserved order. The transition of progenitor cells into terminally differentiated retinal cells is accompanied by repression of progenitor cell-related genes and upregulation of genes specific for retinal cell types. Emerging evidence suggests that histone methylation in the differentiated retinal cells is dynamically regulated and contributes to retinal differentiation in a lineage-specific manner. Proper retinal-terminal differentiation requires precise methylation of H3K9/27 and H3K4, which are predominantly correlated with gene repression and activation, respectively (Iwagawa and Watanabe, 2019; Pova et al., 2021). Previous genome-wide analyses of retinal methylation profiles using chromatin immunoprecipitation sequencing (ChIP-seq) have shown that rod photoreceptor differentiation is accompanied by de novo H3K4me2/3 accumulation around the transcription starting sites (TSS) and gene bodies of a subset of rod photoreceptor-specific genes, which correlates with their increased gene expression (Peng et al., 2012; Ueno et al., 2016). However, the action of H3K4me2/3 on photoreceptor-specific gene activation remains unclear.

Histone methylations can serve as signals for the recruitment of distinct “reader” proteins to alter chromatin accessibility. For instance, H3K4me3, which is enriched at active promoters, can recruit chromatin-modifying factors, such as histone acetyltransferases, resulting in histone acetylation and gene activation. Moreover, H3K4 methylation may contribute to transcription initiation via recruitment of basal transcription machinery or tethering of specific transcription factors to target gene promoters. Detectable H3K4me3 has been found at active enhancers bound by RNA polymerase II (Pol II) (Calo and Wysocka, 2013). However, it is unclear how H3K4me2/3 signals are transduced to transcription machinery that are subsequently recruited to specific photoreceptor genes during retinal development. Previous studies have determined a series of transcription factors that execute essential roles in photoreceptor development and maintenance (Hennig et al., 2008; Swaroop et al., 2010). CRX (cone-rod homeobox protein) is known as a key transcription factor for photoreceptor gene transcription (Furukawa et al., 1997, 1999). A crucial function of CRX is to recruit HAT-containing coactivators to photoreceptor gene chromatin for histone acetylation (Peng et al., 2012; Ueno et al., 2016).
and Chen, 2007). Recent study demonstrated that CRX is required for chromatin remodeling at specific target sites, which undergo retina or neuronal specific activation during photoreceptor differentiation (Ruzycki et al., 2018). CRX is expressed originally in photoreceptor precursors that are localized to the gene-rich euchromatin regions of the rod photoreceptor nuclei (Corbo et al., 2010; Popova et al., 2012; Sоловei et al., 2009), where H3K4me2/3 markers are also highly enriched during retinal development (Rao et al., 2010). It is largely undetermined whether H3K4me2/3 are required for CRX function.

High levels of transcription have been linked to a high degree of H3K79 methylation, which typically peak around the transcription start site (TSS) with diminished levels throughout genes (Bernt et al., 2011; Steger et al., 2008). Importantly, H3K79 methylation has been linked to embryonic development, hematopoiesis, cardiac function, and the development of leukemia (Ljungman et al., 2019; Nguyen and Zhang, 2011), whereas H3K79 methylation in the regulation of retinal photoreceptor maturation and integrity has not been investigated. H3K79 methylation is present in the promoter and coding regions of active genes, suggesting a role for H3K79 methylation in transcription activation and elongation (Wood et al., 2018). Although the mechanism by which H3K79 methylation regulates transcription is largely undetermined, H3K79 may act through recruiting or repelling effector proteins (Vlaming and van Leeuwen, 2016). It has also been suggested that H3K79 methylation may inhibit chromatin localization of repressors, thereby maintaining an open chromatin state with elevated H3K9 acetylation and H3K79 methylation (Chen et al., 2015). It is of note that the patterning of H3K79 methylation and H3K4 methylation shows several similarities in mammalian chromatin (Steger et al., 2008). High H3K4 and H3K79 methylation and H3 acetylation are required for the recognition and binding of c-Myc to target genes (Guccione et al., 2006). Moreover, H2BK123 ubiquitination by Rad6 (ubiquitin-conjugating E2 enzyme) and Bre1 (ubiquitin E3 ligase) is a prerequisite for both H3K4 and H3K79 methylation (Shahbazian et al., 2005); however, whether H3K4 and H3K79 methylation are linked in the transcriptional regulation of photoreceptor genes is still unclear.

Methylation of H3K4 is known to be catalyzed by the SET (Su(var) three to nine, Enhancer-of-zeste, and Trithorax) domain containing histone methyltransferas es (HMT), which are conserved from yeast to humans. In mammalian cells, the MLL (or KMT2) family consists of seven members, including MLL1-5 (KMT2A-2E), SET1A (KMT2F), and SET1B (KMT2G). MLL1 has been reported to play a cell type-specific role in retinal development at multiple levels that differs from other histone modifying enzymes, such as EED and EZH2 in the polycomb repressive complex 2 (RPC2) (Brightman et al., 2018). A recent study showed that SET1A played pivotal roles for the survival and proliferation of retinal progenitors via H3K4 modifications of Uhrf1 (Deng et al., 2021). MLL5 (also known as KMT2E) consists of a single N-terminal SET domain and a plant homeodomain (PHD) zinc finger (Zhang et al., 2017). Although the function of the MLL5 PHD finger as a molecular reader of H3K4me2/3 has been well-documented, the role of MLL5 as an epigenetic modifying factor remains controversial following several reports demonstrating that recombinant MLL5 lacks intrinsic HMT activity. Although the MLL5 splice variant has been reported to exhibit in vitro H3K4 tri-methylation activity (Nin et al., 2015), it remains unclear whether MLL5 possesses cell type or cell context-dependent HMT activity. MLL5 has been reported to be essential for cell cycle progression and genomic stability maintenance (Zhao et al., 2016). Previous studies of MIIS-deficient mice have demonstrated a requirement for MLL5 in hematopoiesis and spermatogenesis, as well as in the immune system (Zhang et al., 2017). Here, we demonstrate that MLL5 is highly expressed in the mouse retina and is essential for visual function. MLL5 facilitates the establishment of proper methylation of H3K4 and H3K79 at photoreceptor gene promoters. Moreover, MLL5 associates with CRX, which subsequently recruits CBP and promotes a permissive chromatin state for photoreceptor gene transactivation.

RESULTS

MLLS is abundantly expressed in the mouse retina

Previous RNA tissue arrays indicated that MLL5 is highly expressed in fetal thymus and kidney and in adult hematopoietic tissues, jejunum, and cerebellum (Emerling et al., 2002). We wondered whether elevated expression of MLL5 is responsible for cell type or cell context-dependent activity. To identify additional MLL5 expression cells or tissues, we searched the Single-cell RNA-seq data of the Single Cell Portal database (https://singlecell.broadinstitute.org/single_cell) in 340 total studies collected by the Broad Institute. Using this approach, we found that 197 studies showed expression of the MLL family genes with different patterns in different cell types. Interestingly, MLL5 expression is the highest compared to other members of the MLL family in all 10 retinal studies.
MLL5 is necessary for visual function of the mouse retina

(A) qPCR analysis of MLL5 transcripts in different mouse tissues (8-week-old) shows significantly higher expression in the retina compared with other tissues (normalized to the average ct values of reference genes 18S rRNA and β-actin). Error bars represent SEM (n = 3).

(B) WB: MLL5 protein level (mouse retinas)
To explore the potential involvement of MLL5 in retinal development and maintenance, we examined its expression pattern in murine retina. Mll5 mRNA was ubiquitously expressed in all examined tissues of adult mice, consistent with previous reports (Emerling et al., 2002), but with highest Mll5 mRNA expression level found in the retina (Figures 1A and S1A). Abundant MLL5 protein expression in the retina was confirmed by immunoblot (Figures 1B and 1C). We then examined MLL5 expression in the retina across various postnatal developmental stages. MLL5 protein was detected in developing retinas at P7 and P21 and at slightly lower levels in adult retinas at P30 and P60 (Figures 1D and 1E). However, we detected no significant differences in Mll5 mRNA expression between developmental time points (Figure S1B), suggesting possible posttranscriptional regulation of MLL5 during retinal development. To investigate the spatial expression of MLL5 in the retina, we assessed Mll5 mRNA expression by in situ hybridization (ISH) with Mll5 antisense probe, and Mll5 sense probe was used as the negative control. Mll5 expression was observed in all nuclear layers of the dorsal retina and ventral retina at the age of P21 (Figures 1F and S1C). Given the abundant expression of Mll5 in the retina, we speculated that Mll5 may play a role in visual function.

**Mll5-deficient mouse exhibit early-onset attenuated electroretinogram (ERG) responses**

To investigate a possible role for Mll5 in visual function of retina, we conducted a morphological analysis of retinas from Mll5-KO mouse, which was generated by removing eight nucleotides from the third coding exon of the Mll5 gene using CRISPR/Cas9 (Zhou et al., 2018). This allele results in a knockout of Mll5 protein by introducing a premature stop codon in exon three because of a frameshift. The absence of Mll5 protein in Mll5-KO retinas was confirmed by immunoblotting with anti-Mll5 antibody (Cheng et al., 2008) (Figure S1D). We analyzed in vivo scotopic (dark-adapted) and photopic (light-adapted) electroretinogram (ERG) responses in control and Mll5-KO mouse at P16. The scotopic ERG was elicited by five different light stimulus intensities (−1.7, −0.3, 0.7, 2.1, and 3.0 log cd s/m²) (Figure 1G). The amplitude of the scotopic a-wave and b-wave, which originate from the activity of rod photoreceptors and rod bipolar cells, respectively, was significantly decreased in Mll5-KO mice at all light stimulus intensities (Figures 1H and 1I). The photopic ERG was also elicited using five different light stimulus intensities (0.7, 1.4, 2.1, 3.0, and 3.3 log cd s/m²) (Figure 1J). The amplitude of the negative a-wave, which represents electrical potentials from cone photoreceptors, was significantly reduced at a high stimulus intensity of 3.3 log cd s/m² in Mll5-KO mice but was unchanged at lower stimulation intensities (Figure 1K). The amplitude of the positive b-wave, which originates from cone bipolar cells activated by cone photoreceptors, was also significantly decreased at higher stimulus intensities (2.1–3.3 log cd s/m²) (Figure 1L). It is noted that Mll5-KO retinas showed more severe loss of both scotopic and photopic b-waves versus the loss of a-waves, indicating that Mll5 depletion may have more impact on the inner layers of the retinal bipolar cells. To determine whether visual decline in Mll5-KO retinas is caused by the Pde6bdr1 mutation, which is a spontaneous lesion common among laboratory inbred mice arising from a murine viral insertion in exon seven of the Pde6b (phosphodiesterase 6b) gene (Chang et al., 2002), we performed DNA sequence analysis of both Mll5-WT and Mll5-KO retinas. Both retinas are confirmed to carry the wild type Pde6b alleles (data not shown). Therefore, Mll5 depletion disrupts both scotopic and photopic ERG.

The change of ERG response of Mll5-KO mouse promotes us to investigate whether these changes were because of disrupted retinal structure. It is of note that Mll5-KO mouse eyeball size is not changed...
compared to MiI5-WT, although MiI5-deficient mouse exhibited systemic abnormalities (Zhou et al., 2018). Hematoxylin-and-eosin (H&E) staining of retinal sections from three-week-old MiI5-HET and MiI5-KO mice revealed normal architectures (Figure S2A). There was also no change of the outer plexiform (OPL) and inner plexiform (IPL), indicated by staining of glutamine synthetase (GS) and protein kinase C alpha (PKCα) which label Müller cells and rod bipolar cells, respectively (Figure S2B). MiI5-KO retinas contained all major retinal cell types with grossly similar cell numbers to that of MiI5 WT retinas (Figure S2C), suggesting that depletion of MiI5 does not affect normal differentiation of the developing retina. To examine whether MiI5 depletion alters photoreceptor development, we performed immunostaining of MiI5 WT and MiI5 KO photoreceptors. Notably, expression of Rho, which is essential for light sensing in rod photoreceptors, was significantly decreased (Figures S2D–S2F). We also examined cone photoreceptor cells by immunostaining with an antibody against green cone opsin (anti-Opn1mw) as well as with fluorescently labeled lectin peanut agglutinin (PNA), which recognizes the outer and inner segments of cone photoreceptors. We observed a lower density of both PNA staining and dorsal retina Opn1mw staining in MiI5-KO mice compared with MiI5 WT mice, although the localization patterns were similar. We then performed terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) staining to compare rates of apoptosis in MiI5 WT and MiI5 KO retinas from P21 mice. A significantly higher number of TUNEL-positive cells (~10-fold) were detected in retinas from MiI5-KO mice compared with those from MiI5 WT (Figures S2G and S2H). We then evaluated the number of photoreceptor nuclei in the outer nuclear layer (ONL). Compared with MiI5 WT retinas that contain 10–11 layers of photoreceptor nuclei in the ONL, MiI5-KO mice at the age of 6 weeks retained only eight to nine layers of photoreceptor nuclei (Figure S2I). Other layers of MiI5-KO retinas had normal cell numbers (data not shown). These data demonstrate that loss of MiI5 induces mild retinal degeneration, consistent with the ERG amplitude reductions, suggesting that MiI5 is important in photoreceptor maintenance.

**Depletion of MiI5 impairs the expression of critical photoreceptor genes**

Because MiI5 depletion did not change the retinal architecture, we then investigated whether the attenuated ERG is caused by defects on retinal gene expression. We examined genome-wide gene-expression profiles of MiI5 WT and MiI5 KO retinas using high-throughput RNA sequencing (RNA-seq). Two bioreplicates of RNA-seq were performed using MiI5 WT and MiI5 KO retinas at P18 (Figure 2A). A total of 133 genes were found to be dysregulated with at least 2-fold differential expression. In MiI5 KO retinas, 82 genes were downregulated and 51 genes were upregulated compared with MiI5 WT retinas. Functional annotation of the differentially expressed genes using gene ontology (GO) analysis (DAVID web tool) showed enrichment of processes related to visual perception, photoreceptor cell development, and photoreceptor maintenance among the downregulated genes, whereas the upregulated genes were enriched for processes related to cell cycle and cell proliferation (Figure 2B and Table S1). Notably, a number of differentially expressed genes between MiI5 KO and MiI5 WT retina are known to be mutated in inherited human retinal diseases or have been shown to be essential for retinal organization and vision function (Figure 2C). qPCR analysis confirmed a significant reduction in mRNA expression of photoreceptor-specific genes, such as Rho, Opn1sw (blue cone opsin), and Opn1mw in MiI5 KO retinas (Figure 2D), whereas the expression of cell-type-specific genes from different retinal neuronal cell types did not significantly change, except for Rlbp1 (Retinaldehyde-binding protein 1) (Figure S3A). Furthermore, MiI5 depletion did not affect the expression of well-known photoreceptor transcription factors, such as CRX, NRL (neural retina-specific leucine zipper), and Nr2e3 (nuclear receptor subfamily two group E member 3) (Figure S3B), and no significant changes were observed in the expression of core transcription factors and signaling proteins essential for progenitor cell maintenance (Figure S3C). These findings suggest that MiI5 promotes retinal maturation and maintenance through regulating genes essential for photoreceptor organization and function and not via induction of altered expression of key retinogenesis regulators.

**MiI5 is required for CRX-mediated photoreceptor gene transactivation**

To investigate whether MiI5 is directly involved in photoreceptor gene transcription, we examined the effect of MiI5 knockdown on Rho transcription using a Rho promoter-based luciferase assay. A region containing the human Rho (HsRho) distal and proximal promoters (positions −1500 to +10) driving a Firefly luciferase gene was cloned into the pGL3 vector and transfected into HEK293T cells together with an internal control plasmid expressing Renilla luciferase. In line with previous studies (Chen et al., 1997; Irie et al., 2015), the HsRho promoter was significantly activated when the transcription factor CRX was introduced, whereas introduction of MiI5 alone showed marginal transactivation of the HsRho promoter. However, we observed a significant decrease in HsRho promoter activity when endogenous MiI5 was depleted using
Figure 2. Gene expression analysis of Mll5-WT and Mll5-KO retinas
(A) RNA-seq heatmap showing expression patterns of 133 dysregulated genes in Mll5-KO retinas at P16. The expression values are Log2 (FPKM) values and are indicated with green or red colors representing lowest to highest expression levels.
(B) Gene ontology (GO) analysis of downregulated and upregulated genes in Mll5-KO retinas showing the top 10 enriched GO terms. The value of –log10 (p value) was calculated to reflect the significance of GO term enrichment.
(C) Heatmap depicts the Log2 (FPKM) values of Mll5-KO affected genes that are involved in human retinal disease, retina development, and visual functions.
(D) Bar chart showing the downregulation of selected photoreceptor genes and genes that were found dysregulated in RNA-seq analysis. qPCR analysis was performed using P16 Mll5-WT and Mll5-KO retinas and was normalized to the expression of Tbp (TATA binding protein) (n = 3). Error bars show the SEM (n = 5). *p < 0.05, **p < 0.01, ***p < 0.001, Student’s t test.
MLL5-shRNA (shMLL5) (Figure 3A). We performed similar assays on the murine Opn1mw (MmOpn1mw) promoter (positions −1441 to −14) and the Opn1sw (MmOpn1sw) promoter (positions −1450 to −11). A significant decrease in the transactivation of both promoters was also observed in the presence of shMLL5 (Figures 3B and S4A). Next, we examined if MLL5 and CRX synergistically regulate the expression of photoreceptor genes. Enhancement of HsRho transactivation was detected when exogenous MLL5 was co-transfected with CRX (Figure 3C); however, the effect was marginal, presumably owing to the preoccupancy of endogenous MLL5 saturating MLL5-binding sites. Similar results were obtained in the transactivation of...
Figure 3. In contrast, the expression of opsin receptor genes. Therefore, the data indicates that MLL5 cooperates in the transactivation of MLL5 knockdown. These data suggest that MLL5 is essential for the transcription of a subset of photoreceptor genes induced by CRX.

MmOpn1mw and MmOpn1sw promoters (Figures 3D and S4B). It is of note that these transient transfections of episomal luciferase may not contain appropriate chromatin (nucleosomal) structure and result in false-positive results. To exclude this possibility, we next investigated if MLL5 is required for photoreceptor gene transcription using 661W. The 661W photoreceptor cell line was derived from mouse retinal tissue and has been widely used as a model for studying macular degeneration (Tan et al., 2004). Previous studies found that 661W cells expressed the majority of markers of cone cell origin (Wheway et al., 2019). It could be multipotent and could be differentiated to a neuronal phenotype (Thompson et al., 2015). Transduction of 661W using lentivirus has been reported (Hamann et al., 2013; Zhou et al., 2021). We transduced Mll5−/− or Mll5−/−Rho−/− or Mll5−/−Rho−/−PS cells with anti-MLL5 or anti-CRX antibodies, which revealed an interaction between endogenous MLL5 and CRX (Figure 4A). To determine which region of MLL5 binds CRX, we expressed three truncated Flag-MLL5 mutants together with HA-CRX in HEK293T cells and performed co-IP using an anti-HA antibody. HA-CRX was shown to bind the central domain (CD) of MLL5, rather than the PHD/SET domain (PS) or the C-terminal domain (CT) (Figures 4B and 4C). A smaller fragment of the MLL5 CD domain (CD4) was then co-expressed with HA-CRX in HEK293T. As shown in Figure 4D, Flag-MLL5-CD4 maintained its ability to bind HA-CRX. In line with this, an MLL5 mutant construct lacking the CD4 region failed to interact with CRX, indicating that MLL5-CD4 is the key binding region for CRX (Figure 4E). We then examined whether the interaction between MLL5-CD4 and CRX is essential for CRX-mediated transactivation. We incubated 661W (CRX OE) cells with siMLL5 for 16 h to knockdown endogenous MLL5 and then ectopically introduced RNAi-resistant full-length MLL5 (MLL5-FL) or an RNAi-resistant CD4 domain deletion construct (MLL5-ΔCD4) using lentivirus particles. Noninfected 661W cells were eliminated by puromycin selection. MLL5 knockdown reduced Rho transcription in 661W (CRX OE) cells, which could be rescued by MLL5-FL overexpression; however, transcription could not be rescued using MLL5-ΔCD4 (Figure 4F). These data demonstrate that CRX binds the CD4 domain of MLL5, and this interaction is required for photoreceptor gene transactivation.

MLL5 interacts with CRX via its CD4 domain

The importance of MLL5 in CRX-mediated photoreceptor gene transactivation led us to investigate whether MLL5 interacts with CRX directly. Thus, we conducted co-immunoprecipitation (co-IP) studies in retinal lysates from P18 Mll5−/− mice using anti-MLL5 or anti-CRX antibodies, which revealed an interaction between endogenous MLL5 and CRX (Figure 4A). To determine which region of MLL5 binds CRX, we expressed three truncated Flag-MLL5 mutants together with HA-CRX in HEK293T cells and performed co-IP using an anti-HA antibody. HA-CRX was shown to bind the central domain (CD) of MLL5, rather than the PHD/SET domain (PS) or the C-terminal domain (CT) (Figures 4B and 4C). A smaller fragment of the MLL5 CD domain (CD4) was then co-expressed with HA-CRX in HEK293T. As shown in Figure 4D, Flag-MLL5-CD4 maintained its ability to bind HA-CRX. In line with this, an MLL5 mutant construct lacking the CD4 region failed to interact with CRX, indicating that MLL5-CD4 is the key binding region for CRX (Figure 4E). We then examined whether the interaction between MLL5-CD4 and CRX is essential for CRX-mediated transactivation. We incubated 661W (CRX OE) cells with siMLL5 for 16 h to knockdown endogenous MLL5 and then ectopically introduced RNAi-resistant full-length MLL5 (MLL5-FL) or an RNAi-resistant CD4 domain deletion construct (MLL5-ΔCD4) using lentivirus particles. Noninfected 661W cells were eliminated by puromycin selection. MLL5 knockdown reduced Rho transcription in 661W (CRX OE) cells, which could be rescued by MLL5-FL overexpression; however, transcription could not be rescued using MLL5-ΔCD4 (Figure 4F). These data demonstrate that CRX binds the CD4 domain of MLL5, and this interaction is required for photoreceptor gene transactivation.

MLL5 and CRX co-occupy on Rho promoter

The critical role of MLL5 in CRX-mediated photoreceptor gene transactivation (Figure 3) and the physical interaction between MLL5 and CRX (Figure 4) raised the possibility that MLL5 is required for efficient or stable recruitment of CRX to photoreceptor gene promoters. A number of studies have shown that dysregulation of Rho underlies a spectrum of inherited human retinal disease; therefore, we focused our mechanistic studies on the regulation of the Rho gene. We performed qChIP (Quantitative ChIP) analysis on Mll5−/− or Mll5−/−Rho−/− mouse retinas using four primer pairs at intervals of approximately 400 bp, corresponding to different regions of the Rho promoter locus. Primer pairs spanning the 3′-untranslated region (3′UTR) of the Rho gene were used as internal control. Consistent with previous studies (Corbo et al., 2010; Peng and Chen, 2007), CRX was enriched on the Rho promoter in Mll5−/− WT retinas, especially at a region immediately upstream of the TSS (primer pair −219 to +46) and −1.5 kb upstream of the TSS, albeit at relative lower levels (primer pair −684 to −443, −1150 to −814 and −1500 to −1311). Notably, CRX occupation on the Rho promoter decreased more than 2-fold in Mll5−/− WT retinas, indicating that MLL5 enhances the association of CRX with the Rho promoter (Figure 5A). A requirement for CRX in the recruitment of CBP to Opn1 promoters has been reported previously in murine retina and Y79 retinoblastoma cells (Peng and Chen, 2007). CBP has intrinsic histone acetyltransferase activity targeted to histones H2A, H2B, H3, and H4 (Peterson and Laniel, 2004). Consistent with these findings, MLL5 depletion also reduced CBP accumulation at the Rho promoter. H3Ac and RNA polymerase II recruitment on the Rho promoter in Mll5−/− WT retinal tissue was reduced by more than 2-fold compared with Mll5−/− WT retinal tissue.
Figure 5A. As previously reported (Peng and Chen, 2007), CRX, CBP, and H3Ac did not accumulate at the 3′UTR of the Rho gene. We also detected CRX, CBP, and H3Ac recruitment on the promoter of Collagenase gene. Collagenase gene is a non-photoreceptor-specific gene, and previous study has shown that it cannot

Figure 4. MLL5 interacts with CRX via its CD4 domain

(A) Interaction between endogenous MLL5 and CRX. Equivalent amounts of retinal lysates from MII5-WT or MII5-KO mice were immunoprecipitated with anti-MLL5 antibody, anti-CRX or normal rabbit IgG, followed by western blotting detection.

(B) Schematic representation of MLL5 truncated mutants. PS, PHD/SET domain; CD, central domain; CT, C-terminal domain. ΔCT4, deleted CT4 domain.

(C) MLL5 central domain (MLL5-CD) bind to CRX. HEK293T cells expressing HA-CRX together with Flag-MLL5-PS, Flag-MLL5-CD, or Flag-MLL5-CT were subjected to immunoprecipitation with the indicated antibodies followed by western blotting detection.

(D) Interaction between MLL5-CD4 and CRX. HEK293T cells expressing HA-CRX and Flag-MLL5-CD4 were immunoprecipitated with anti-HA antibodies and detected by anti-Flag and anti-HA antibodies.

(E) Depletion of MLL5 CD4 compromises MLL5 interaction with CRX. HEK293T cells expressing HA-CRX along with Flag-MLL5-FL or Flag-MLL5-ΔCD4 were immunoprecipitated with anti-HA and anti-Flag antibodies followed by western blotting detection.

(F) MLL5-ΔCD4 was less potent than MLL5-WT at rescuing transcription of Rho. qPCR analysis of Rho transcript in 661W (CRX OE) cells transfected with MLL5-siRNA (siMLL5) followed by transduction with the indicated lentivirus. Rho expression was normalized to the expression of Tbp. Error bars represent SEM (n = 3), **p < 0.01, Student’s t test. Results in (A–F) are representative of at least three experimental repeats.
Figure 5. MLL5 and CRX co-occupy the Rho promoter
(A) MLL5 depletion leads to reduced binding of CRX, CBP, and Pol II (RNA Polymerase II), as well as decreased H3Ac (H3 acetylation) at the Rho promoter. qChIP (Quantitative ChIP) analysis using P18 Mll5-WT and Mll5-KO retinas with antibodies against CRX, CBP, Pol II, acetylated H3 (H3Ac), or normal rabbit IgG. Immunoprecipitated DNA fragments were analyzed by qPCR for the promoter region and 3′ UTR regions of Rho gene. All data shown are representative qChIP and presented as % of input chromatin. Error bar shows mean SEM (n = 3 from six retinas). **p < 0.01, ***p < 0.001, Student’s t test.
H3K79me2 was also detected on Rho Mll5 in detected H3K4/K79 methylation on on Rho genome-wide analysis of H3K4 methylation in retina (Popova et al., 2012). Notably, H3K4me2/3 accumulation retinas, corresponding to its active expression, as well as in the gene coding regions, consistent with a levels of H3K4me3 as in CreNeg control retinas (Brightman et al., 2018). We then detected histone methylations MLL5 knockdown 661W cells. This is different from ure 6 A), whereas global levels of H3K4me3 and H3K79me2 were reduced in 661W (EV OE) to particular regions targeted by MLL5. Cells infected with Dam-control lentivirus served as a control for back-(CRX OE) cells. Genomic DNA was then isolated and processed to amplify methylated DNA, corresponding and this binding is further enhanced by CRX. Collectively, these data suggest that MLL5 and CRX are co-re-
generate significantly increased relative methylation on the promoter of Collagenase but not in the vicinity of the Rho promoter. As an alternative, we employed DNA adenine methyltransferase identification (DamID), a well-estab-
lished assay for measuring protein-DNA interactions (Vogel et al., 2007), which has previously been used to determine the genomic distribution of MLL5 in C2C12 cells (Ali et al., 2013). Briefly, MLL5 was fused to the N-terminal of the Escherichia coli’s DNA adenosine methyltransferase (Dam) and transduced into control or 661W (CRX OE) cells. Genomic DNA was then isolated and processed to amplify methylated DNA, corresponding to particular regions targeted by MLL5. Cells infected with Dam-control lentivirus served as a control for background DNA methylation. As expected, only a negligible recruitment of MLL5-Dam was observed at the silent immunoglobulin heavy chain enhancer (Buas et al., 2010), which was used to normalize the data. 661W (EV OE) cells expressing MLL5-Dam gave rise to more than 3-fold relative increase in methylation in the Rho promoter but not in the vicinity of the Rho 3’UTR (Figure 5C). Methylation at the Rho distal and proximal promoter regions was further enhanced in 661W (CRX OE) cells transduced with MLL5-Dam (Figure 5C). MLL5 binding on the promoter of Collagenase was also examined by Dam-ID. As expected, 661W cells infected with MLL5-Dam did not generate significantly increased relative methylation on the promoter of Collagenase gene (Figure 5D). These data indicate that MLL5 associates specifically with the Rho promoter, primarily at ~1.5 kb upstream of the TSS, and this binding is further enhanced by CRX. Collectively, these data suggest that MLL5 and CRX are co-re-
recruited to the Rho promoter interdependently.

**MLL5 is critical for the methylation of histone H3K4 and H3K79 on the Rho promoter**

Since it has been suggested that MLL5 might affect H3K4 methylation indirectly by affecting the expression of LSD1 (lysine-specific histone demethylase 1), and SET7/9 in C2C12 murine myoblast cells (Sebastian et al., 2009). We asked whether MLL5 depletion affects histone methylations. Immunoblot analysis revealed similar levels of genome-wide H3K4me1, H3K4me2, H3K9me2, and H3K36me2 in Mll5-WT and Mll5-KO retinas (Figure 6A), whereas global levels of H3K4me3 and H3K79me2 were reduced in Mll5-KO retinas at P18 and in Mll5 knockdown 661W cells. This is different from Mll1 conditionally knocked out retinas which displayed similar levels of H3K4me3 as in CreNeg control retinas (Brightman et al., 2018). We then detected histone methylations on Rho promoter by qChIP analysis. We found an enrichment of H3K4me2/3 on the Rho promoter in Mll5-WT retinas, corresponding to its active expression, as well as in the gene coding regions, consistent with a genome-wide analysis of H3K4 methylation in retina (Popova et al., 2012). Notably, H3K4me2/3 accumulation on Rho promoter was reduced by more than 2-fold in Mll5-KO retinas, which may contribute to Rho repression in Mll5-KO retinas; however, MLL5 depletion did not affect the H3K4me2/3 level on Rho gene 3’ UTR (Figure 6B). H3K79me2 was also detected on Rho promoter, which was reduced in Mll5-KO retinas (Figure 6B). We also detected H3K4/K79 methylation on Collagenase gene in Mll5-WT and Mll5-KO retinas, and found that there was no

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**Figure 5. Continued**

(B) Recruitment of CRX, CBP, Pol II, and acetylated H3 at the Rho promoter are decreased in 661W (CRX OE) cells when the ΔCD4 domain of MLL5 was deleted. 661W (CRX OE) cells, depleted of endogenous MLL5s using siRNA, were further transduced with MLL5-WT or MLL5-ΔCD4 by lentivirus. qChIP analysis was performed with antibodies against CRX, CBP, Pol II, H3Ac, or normal rabbit IgG. Immunoprecipitated DNA fragments were analyzed by qPCR for the region of the Rho promoter and 3’UTR. Representative qChIP data of at least three experimental repeats are shown and presented as % of input chromatin. Error bar represents SEM (n = 3). **p < 0.01. ***p < 0.001, Student’s t test.

(C and D) CRX enhances the recruitment of MLL5 at the Rho promoter but not at the Collagenase promoter. 661W (EV OE) cells and 661W (CRX OE) cells were transduced with MLL5-Dam or Dam-control by lentivirus followed by G418 selection to remove noninfected cells. Genomic DNA was then harvested and subjected to a DamID protocol, followed by qPCR using primers in the proximity to the promoter regions of Rho, 3’UTR, within the enhancer of immunoglobulin heavy chain (IgH), or the promoter regions of Collagenase as negative control. Data are presented as ratios of the MLL5-Dam qPCR signal to the Dam-only signal. Representative results of at least three experimental repeats are shown. Error bar represents SEM (n = 3), *p < 0.05. **p < 0.01, Student’s t test.

To determine whether decreased recruitment of CRX/CBP is a consequence of the attenuated interaction between MLL5 and CRX, we depleted endogenous MLL5 and then introduced RNAi-resistant MLL5-FL or MLL5-ΔCD4 into 661W (CRX OE) cells. Consistent with our qChIP results in Mll5-WT retinas, the Rho promoter displayed CRX accumulation, CBP binding, H3 acetylation, and RNA polymerase II recruitment in 661W cells expressing MLL5-FL, although these effects were significantly decreased in MLL5-ΔCD4 expressing 661W cells (Figure 5B). Collectively, these data indicate that MLL5 play a role in the recruitment or stabilization of CRX/ CBP to the Rho promoter, which is dependent on the direct interaction between CRX and MLL5.

Next, we asked whether CRX could alter MLL5 recruitment to chromatin. A lack of ChIP-grade antibodies capable of detecting mouse MLL5 meant that we could not investigate MLL5 occupancy on the Rho promoter in vivo. As an alternative, we employed human adenine methyltransferase identification (DamID), a well-establish-
ed assay for measuring protein-DNA interactions (Vogel et al., 2007), which has previously been used to determine the genomic distribution of MLL5 in C2C12 cells (Ali et al., 2013). Briefly, MLL5 was fused to the N-terminal of the Escherichia coli’s DNA adenosine methyltransferase (Dam) and transduced into control or 661W (CRX OE) cells. Genomic DNA was then isolated and processed to amplify methylated DNA, corresponding to background DNA methylation. As expected, only a negligible recruitment of MLL5-Dam was observed at the silent immunoglobulin heavy chain enhancer (Buas et al., 2010), which was used to normalize the data. 661W (EV OE) cells expressing MLL5-Dam gave rise to more than 3-fold relative increase in methylation in the Rho promoter but not in the vicinity of the Rho 3’UTR (Figure 5C). Methylation at the Rho distal and proximal promoter regions was further enhanced in 661W (CRX OE) cells transduced with MLL5-Dam (Figure 5C). MLL5 binding on the promoter of Collagenase was also examined by Dam-ID. As expected, 661W cells infected with MLL5-Dam did not generate significantly increased relative methylation on the promoter of Collagenase gene (Figure 5D). These data indicate that MLL5 associates specifically with the Rho promoter, primarily at ~1.5 kb upstream of the TSS, and this binding is further enhanced by CRX. Collectively, these data suggest that MLL5 and CRX are co-re-
recruited to the Rho promoter interdependently.

Figure 5. Continued
Figure 6. MLL5 is critical for the methylation of histone H3K4 and H3K79 on the Rho promoter

(A) Representative western blotting of at least three experimental repeats shows reduced global H3K4me3 and H3K79me2 levels in Mll5-KO retinas. Equivalent amounts of protein (5 μg) were loaded for each panel.

(B) MLL5 depletion leads to decreased H3K4me2/3 and H3K79me2 levels at the Rho promoter. qChIP (Quantitative ChIP) analysis using P18 Mll5-WT and Mll5-KO retinas with antibodies against H3K4me2, H3K4me3, H3K79me2, or normal rabbit IgG. Immunoprecipitated DNA fragments were analyzed by qPCR for the promoter region and gene coding region of Rho gene. All data shown are representative qChIP and presented as % of input chromatin. Error bar shows mean SEM (n = 3 from six retinas). **p < 0.01, ***p < 0.001, Student’s t test.

(C) qPCR analysis shows that the MLL5-W141A mutant failed to rescue Rho expression in MLL5-KD 661W (CRX OE) cells. 661W (CRX OE) cells were transfected with MLL5-siRNA (siMLL5) for 16 h and then infected with lentivirus particles expressing RNAi-resistant Flag-MLL5 or RNAi-resistant domain mutant MLL5 constructs. Noninfected cells were eliminated by puromycin selection. Overexpression of MLL5 and CRX was determined by western blotting. Error bars represent SEM (n = 3).

(D and E) qChIP analysis shows that MLL5 PHD finger mutation did not affect the recruitment of H3K79me2 but caused decreased recruitment of H3K4me2/3, CRX, CBP, Pol II, and H3Ac to the Rho promoter. 661W (CRX OE) cells were transfected with siMLL5 and then transduced with MLL5-WT, -W141A, or -T440A.
H3K4me3 and H3K79me2 on Collagenase gene (Figure S6). High H3K4/K79 methylation has been associated with preengaged basal transcription machinery (Guccione et al., 2006). We found that RNA polymerase II bound on Rho gene but not Collagenase gene in retinas, and this binding was reduced in Mll5-KO retinas at P18 (Figures 5A and S6). Collectively, these data suggest MLL5 depletion reduced methylation of H3K4 and H3K79 on Rho gene, which may contribute to reduced RNA polymerase II binding on Rho gene and Rho gene repression in Mll5-KO retinas.

The MLL5 PHD finger is critical for CRX recruitment to Rho promoter

It has been demonstrated that MLL5 and RNA polymerase II co-occupy promoters that display H3K4me3, suggesting that MLL5 may have the capacity to read H3K4me3 modification, and recognition of the H3K4me3 can facilitate the recruitment of MLL5 to active transcription chromatin regions. Further studies indicated that MLL5 binds strongly and specifically to histone H3K4me3 through its PHD finger, and substitution of W141 with an alanine completely abolished the MLL5 PHD finger and H3K4me3 interaction (Ali et al., 2013; Lemak et al., 2013; Zhang et al., 2017). We then examined whether the MLL5 PHD finger has a role in Rho transactivation. We first dissected to find whether mutations in the PHD finger could alter Rho transactivation. We depleted endogenous MLL5 and then transduced exogenous RNAi-resistant wild-type-MLL5 (MLL5-WT) or MLL5-W141A mutants into 661W (CRX OE) cells. Notably, MLL5-WT protein restored Rho transcription, whereas the MLL5-W141A mutant, which disrupts MLL5 binding to H3K4me2/3, failed to do so (Figure 6C). T440 is a conserved site of O-GlcNAcylation in two short forms of MLL5 (Ding et al., 2015; Nin et al., 2015). Rho transactivation was not affected by transduction of the MLL5-T440A mutant, indicating that glycosylation is not involved in this interaction (Figure 6C). Taken together, our results suggest that the MLL5 PHD finger is essential for Rho transactivation.

To assess how the PHD finger functions on Rho gene transactivation, we sought to determine whether MLL5 recruitment to chromatin through PHD finger is required for the recruitment of CRX-CBP to activate Rho transcription. We first checked whether the MLL5 PHD finger is required for H3K4me3 and H3K79me2 on the Rho promoter. qChIP analysis in endogenous MLL5-depleted 661W (CRX OE) cells revealed high levels of H3K4me2/3 at the Rho promoter in the presence of exogenous MLL5-WT, which was reduced by more than 2-fold when exogenous MLL5-W141A mutant was transduced. Importantly, PHD domain mutation did not affect the H3K79me2 at the Rho promoter. Expression of the glycosylation site mutant exogenous MLL5-T440A did not alter H3K4me2/3 and H3K79me2 at the Rho promoter (Figure 6D). We next detected whether PHD finger mutation affects CRX-CBP binding to the Rho promoter by qChIP. As expected, CRX and CBP were recruited to the Rho promoter in the presence of exogenous MLL5-WT. However, enrichment of both molecules was reduced by more than 2-fold in the presence of MLL5-W141A mutant versus MLL5-WT. Reduced RNA polymerase II binding on the Rho promoter was also detected in the cells expressing MLL5-W141A mutant, but not in MLL5-T440A-expressing cells (Figure 6E). Collectively, these results demonstrate that MLL5 directly influences CRX-CBP recruitment to the Rho promoter, possibly through recognition of H3K4me2/3 by its PHD finger.

DISCUSSION

Histone modifications and transcription factors are important components of transcriptional regulation. Despite increasing numbers of studies demonstrating that H3K4 methylation and CRX are both involved in photoreceptor gene transactivation and maturation, whether H3K4 methylation links to transcription factor recruitment is undetermined. Our data indicate that an intact PHD finger is essential for the MLL5 function on photoreceptor gene transcription, with mutations of the PHD finger leading to compromised CRX-CBP assembly. In Mll5-KO retinas, loss of MLL5 leads to decreased H3K4/K79 methylation and impaired CRX-CBP occupancy on photoreceptor gene promoters, reducing histone acetylation and photoreceptor gene transcription, which results in impaired retinal function (Figure 7A). Moreover, we observed that MLL5 interacts with CRX via its CD4 domain, and the interdependent recruitment of MLL5 and CRX to the Rho promoter is essential for Rho activation. The MLL5 PHD domain may anchor MLL5 to chromatin through recognition of H3K4me2/3, which is a prerequisite for CRX recruitment to chromatin. CRX then assembles CBP, which catalyzes histone H3 acetylation, facilitating RNA polymerase II transcription of Rho.
Hence, our study suggests that MLL5 acts as a cofactor for CRX to generate a permissive chromatin state for photoreceptor gene transcription and provides a new perspective on how photoreceptor-specific gene expression is established and maintained in the retina.

MLL5’s role in coupling CRX and CBP with H3K4 methylation adds another layer of complexity to the spatiotemporal regulation of photoreceptor genes. Chromatin remodeling complexes and HAT-containing coactivators have been implicated in photoreceptor gene transcriptional activation, in concert with CRX (Hennig et al., 2008; Swaroop et al., 2010). Recent studies revealed that Rho activation is accompanied by de novo accumulation of H3K4me2/3, observed as early as P2 (Popova et al., 2012; Swaroop et al., 2010; Ueno et al., 2016). Notably, during mouse photoreceptor development, expression of CRX and CBP is present from the embryonic stage, whereas binding to the Rho promoter is only detected after P2 (Peng and Chen, 2007), suggesting that H3K4 methylation coincides with the establishment of the key basal transcription machinery required for Rho transcription. However, CRX itself is incapable of H3K4me2/3 recognition and requires assistance from MLL5, evidenced.
by reduced CRX recruitment in the absence of MLL5 (Figure 5A). Further investigations into the co-occupancy of MLL5 and CRX and the co-occupancy of MLL5 and H3K4me2/3 on photoreceptor gene promoters using sequential ChIP analysis would be valuable once ChIP-grade anti-MLL5 antibodies become available.

To examine the spatial expression pattern of MLL5 in the murine retina, we performed ISH with an Mll5 antisense probe. We also tried immunolocalization of MLL5 with two in-house generated and several commercially available anti-MLL5 antibodies. However, they are suitable for Western blotting and immunofluorescent staining in cell lines, none of them were suitable for retinal cryostat sections and paraffin sections because of high background and poor specificity. It would be interesting to dissect the spatial and temporal expression of MLL5 during retinal development if antibodies for immunohistochemistry of retinal sections are available.

Previous studies have involved MLL5 in gene transcription regulation, although the underlying mechanism remains elusive. A study by Sebastian et al. showed that MLL5 negatively regulated CCNA2 gene expression by binding to its proximal promoter in C2C12 cells (Sebastian et al., 2009). Another study on HsMLL5a, one of MLL5 isoforms, demonstrated that HsMLL5a is recruited to E2F1-responsive promoters by HCF1 in HEK293T and HeLa cells, resulting in activation of E2F1 target genes, such as CCNA2, CDC2, and CDC6 (Zhou et al., 2013). Interestingly, both studies reported decreased H3K4me2/3 on CCNA2 promoter but opposite regulation of CCNA2 gene expression. We observed that MLL5 depletion reduced H3K4 methylation on the Rho promoter, whereas the underlying mechanism is still unclear. These data indicate that MLL5 may regulate gene transcription directly or indirectly in a cell-type/context-dependent manner (Zhang et al., 2017).

A compelling aspect of this study is that MLL5 appears to be a reader of H3K4me on Rho gene which is required for the recruitment of CRX-CBP and histone acetylation. Intriguingly, putative MLL5 orthologues, including UpSET, SET3/4, and SETD5 have been reported, as part of a histone deacetylase-containing complex that is recruited to transcribed genes to restrict histone acetylation and prevent inappropriate gene expression (Zhang et al., 2017); however, it is unclear how these orthologues are recruited to chromatin. Our results suggest that MLL5 potentially modulates photoreceptor-specific histone acetylation in a transcription factor-dependent manner through facilitating the recruitment of histone acetyltransferase instead of histone deacetylase. An earlier study also showed a similar gene activation regulation mechanism where Pax6-mediated recruitment of histone methyltransferase to lens gene chromatin contributes to the binding of histone acetyltransferase (HAT) p300 and the generation of enhancer-specific patterns of core histone modifications (Sun et al., 2016). Our data also demonstrate that H3K79 methylation is reduced in the absence of MLL5 in retinal cells.

In vitro studies have shown that Dot1L is the only known methyltransferase capable of H3K79 methylation (Feng et al., 2002), although there is evidence that another enzyme, RE-IIBP, can methylate H3K79 as well (Woo Park et al., 2015). However, we did not detect any interaction between MLL5 and DOT1L. The expression of DOT1L was also not affected in Mll5 KO retinas (data not shown). Further investigations into the recruitment of DOT1L or RE-IIBP on photoreceptor gene promoters may help explain why Rho promoter has lower H3K79me2 in Mll5 KO retinas.

A previous study characterizing the functions of the SET1/MLL family in the context of planarian regeneration demonstrated that Smed-set1, -mll1/2, -trr-1, and -mll5-2, which are clustered with vertebrate SET1, MIL1/2, MIL3/4, and MIL5, respectively, are required for visible photoreceptor regeneration (Hubert et al., 2013). Recent studies demonstrated that MLL1 plays an essential role in the neural development of zebrafish (Huang et al., 2015) and is indispensable for murine retinal neurogenesis and function development, although mechanistic study did not show gross H3K4 methylation change in Mll1 depleted retinas (Brightman et al., 2018). Thus, it would be informative to study whether additional MLL family members play a role in the H3K4 methylation during retinal development.

Moreover, we found that Mll5 deficiency induced considerable increase of photoreceptor death, in concert with photoreceptor loss, which we could not determine from when did this start because of perinatal lethality of Mll5-KO mouse. Mll5 gene transcript was detected in both the inner and outer retinas, and Mll5-KO retinas showed more severe loss of both scotopic and photopic b-waves versus the loss of a-waves. This suggests that Mll5 may not only function in photoreceptor cells in the ONL but also in the inner nuclear layer, such as bipolar cells and Muller cells. However, Mll5 depletion only affected the cell number of photoreceptor cell layers but not other neural layers and glial cells. The expression and localization of protein kinase C alpha (PKCa) which labels rod bipolar cells also showed normal expression. It is thus largely undetermined whether Mll5 also affects
retinal functions through regulation of bipolar cells. Interestingly, MLL5 gene transcript was also detected in Müller cells. Although MLLS depletion did not significantly reduce the expression of Glu1 (Glutamine synthetase gene), the expression of Rlbp1 was decreased in Mi5-KO retina. Rlbp1 gene is a key component of the retinal visual cycle in Müller cells that helps cone cells to function in high light intensities (Xue et al., 2015). It would be valuable to determine whether MLL5 also contributes to cone-driven vision and light responses through regulation of Rlbp1. Further discovery of MLL5 functions in other retinal neuronal cell types and in retinal degeneration would greatly advance our knowledge of MLL family members-mediated retinal development and maintenance.

Because mutations of Crx and Rho have been associated with human retinal disease, MLL5’s interaction with CRX and its role in Rho regulation suggest that MLL5 depletion may also contribute to retinal disease pathogenesis. Further exploration of how an epigenetic regulator such as MLL5 and DOT1L take part in retinal development and maintenance will further our understanding on the fundamental mechanism of retinogenesis and clarify the role of epigenetic deregulation in retinal disease progression.

Limitations of the study
In this study, we showed MLL5 is required for photoreceptor gene transactivation. MLL5 expression was detected in both the inner and outer retinas, suggesting it functions not only in photoreceptors but also in other cell types. However, the Mi5-KO retina showed normal architecture. The expression of other cell-type specific genes from different retinal neuronal cell types also did not significantly change in the absence of MLL5, except for Rlbp1, an essential gene of Müller cells, which suggests that MLL5 may also have function in Müller cells. Further study will be required to better characterize MLL5 function in the inner nuclear layer.

STAR* METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.104058.

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AUTHOR CONTRIBUTIONS
X-M.Z., L-W.D., and Z-B.J. designed the research. X-M.Z., B-W.Z., L.X., H.W., A.S., P-P.Z., M.D., X-Y.W., W-J.X., and Y.Z. performed the research. X-M.Z., B-W.Z., L.X., and H.W., analyzed the data. X-M.Z. and L-W.D. wrote the manuscript.

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The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| MLL5 Rabbit PolyAb  | Zhao et al. (2016) | N/A        |
| α-tubulin Mouse mAb | Santa Cruz | Cat# sc-5286, RRID:AB_628411 |
| GAPDH Rabbit PolyAb | Santa Cruz | Cat# sc-25778, RRID:AB_10167668 |
| H3K4me1 Rabbit PolyAb | Abcam | Cat# ab8895, RRID:AB_306847 |
| H3K4me2 Rabbit mAb  | Abcam | Cat# ab32356, RRID:AB_732924 |
| H3K4me3 Mouse mAb   | Abcam | Cat# ab1012, RRID:AB_442796 |
| H3 Rabbit mAb       | Cell Signaling Technology | Cat# 12648, RRID:AB_2797978 |
| PKCs Mouse mAb      | Santa Cruz | Cat# sc-8393, RRID:AB_628142 |
| Rho Mouse mAb       | Abcam | Cat# sc54177, RRID:AB_304874 |
| Opn1mw Goat PolyAb  | Santa Cruz | Cat# sc-22117, RRID:AB_2300956 |
| Flag Mouse mAb      | Sigma-Aldrich | Cat# F3165, RRID:AB_259529 |
| Flag Rabbit PolyAb  | Sigma-Aldrich | Cat# F7425, RRID:AB_439687 |
| HA Mouse mAb        | Sigma-Aldrich | Cat# H9658, RRID:AB_260092 |
| β-Actin Rabbit PolyAb | AbClonal | Cat# AC006, RRID:AB_2768236 |
| CRX Rabbit PolyAb   | Santa Cruz | Cat# sc-30150, RRID:AB_2276566 |
| CRX Mouse mAb       | Abnova | Cat# H00001406-M02, RRID:AB_606098 |
| Calbindin Mouse mAb | Sigma-Aldrich | Cat# C9848, RRID:AB_476894 |
| Pax6 Rabbit PolyAb  | Millipore | Cat# AB2237, RRID:AB_1587367 |
| Chx10 Mouse mAb     | Santa Cruz | Cat# sc-365519, RRID:AB_10842442 |
| RNA polymerase II Rabbit PolyAb | Santa Cruz | Cat# sc-9001, RRID:AB_2268548 |
| Cbp Rabbit PolyAb  | Santa Cruz | Cat# sc-369, RRID:AB_631006 |
| H4Ac Rabbit PolyAb  | Millipore | Cat# Ab 06866, RRID:AB_310270 |
| GFP Mouse mAb       | Abcam | Cat# ab1218, RRID:AB_298911 |
| normal mouse IgG    | Santa Cruz | Cat# sc-2025, RRID:AB_737182 |
| normal rabbit IgG   | Santa Cruz | Cat# sc-2027, RRID:AB_737197 |
| Anti-rabbit IgG, HRP | Abcam | Cat# ab6721; RRID:AB_955447 |
| Anti-mouse IgG, HRP | Abcam | Cat# ab6728; RRID:AB_955440 |
| Goat anti-mouse IgG (H + L), Alexa Fluor 488 | Molecular Probes | Cat# A-11029, RRID:AB_138404 |
| Goat anti-rabbit IgG (H + L), Alexa Fluor 488 | Molecular Probes | Cat# A-11008, RRID:AB_143165 |
| Goat anti-rabbit IgG (H + L), Alexa Fluor 568 | Molecular Probes | Cat# A-11011, RRID:AB_143157 |
| Goat anti-mouse IgG (H + L), Alexa Fluor 568 | Molecular Probes | Cat# A-11031, RRID:AB_144696 |
| Donkey anti-goat IgG (H + L), Alexa Fluor 568 | Molecular Probes | Cat# A-11057, RRID:AB_142581 |
| **Chemicals, peptides, and recombinant proteins** | | |
| FITC conjugate PNA  | Sigma-Aldrich | L7381 |
| Polybrene           | Sigma-Aldrich | TR-1003-G |
| Geneticin           | Gibco | 10131035 |
| Puromycin           | Gibco | A1113803 |
| TRIzol             | Invitrogen | 155967-026 |
| Protease Plus       | ACD | 322331 |
| MLL5 RNAscope probe | ACD | 575421 |

(Continued on next page)
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Critical commercial assays** |        |            |
| SuperScript III RT-PCR system | Invitrogen | 18080-051 |
| iTaq universal SYBR green supermix | Bio-Rad | 172-5120 |
| One Shot™ Gateway PCR Cloning System | Invitrogen | 12535029 |
| Pfx DNA polymerase | Invitrogen | 11708 |
| RNAiMAX Lipofectamine | Invitrogen | 13667-150 |
| True Blot anti mouse IgG IP beads | eBioscience | 00-8811-25 |
| True Blot anti rabbit IgG IP beads | eBioscience | 00-8800-25 |
| RNAscope 2.5 HD Detection Kit-Brown | ACD | 322310 |
| RNeasy mini kit | Qiagen | 74104 |
| Dual-luciferase assay system | Promega | E1910 |
| EpiQuikTM chromatin immunoprecipitation kit | Epigentek | P-2002 |
| DNeasy Tissue kit | Qiagen | 69504 |
| Advantage cDNA polymerase mix | Clontech | 639105 |
| **Deposited data** |        |            |
| Unprocessed western blot data and processed RNA-seq data | Mendeley Data | https://doi.org/10.17632/8vpy42r34y.1 |
| Raw RNA-seq datasets | GEO | GSE196935 |
| **Experimental models: Cell lines** |        |            |
| 661W cell line | Tan et al. (2004) | RRID:CVCL_6240 |
| HEK293FT cell line | ATCC | RRID:CVCL_6911 |
| HEK293T cell line | ATCC | RRID:CVCL_0063 |
| **Experimental models: Organisms/strains** |        |            |
| Mll5-KO mouse | Zhou et al. (2018) | N/A |
| **Recombinant DNA** |        |            |
| Plasmid: pLgw-V5-EcoDam | Addgene | 59210 |
| Plasmid: pLgw-RFC1-V5-EcoDam | Addgene | 59205 |
| Plasmid: pLgw-MLLS-V5-EcoDam | This paper | N/A |
| Plasmid: pGL3-basic | Promega | U47295 |
| Plasmid: pGL3-basic-Rho | This paper | N/A |
| Plasmid: pGL3-basic-Opn1mw | This paper | N/A |
| Plasmid: pGL3-basic-Opn1sw | This paper | N/A |
| Plasmid: pEF6 | Invitrogen | K9610-20 |
| Plasmid: pEF6-MLLS-PS | Zhao et al. (2016) | N/A |
| Plasmid: pEF6-MLLS-CD | Zhao et al. (2016) | N/A |
| Plasmid: pEF6-MLLS-CT | Zhao et al. (2016) | N/A |
| Plasmid: pEF6-MLLS-CD4 | Zhao et al. (2016) | N/A |
| Plasmid: pEF6-MLLS-ΔCD4 | This paper | N/A |
| Plasmid: pLenti-CMV-Puro | Addgene | 17448 |
| Plasmid: pLenti-CMV-Puro-MLLS-ΔCD4 | This paper | N/A |
| Plasmid: pLenti-puro-Flag-MLLS | This paper | N/A |
| Plasmid: pLenti-puro-dest | Addgene | 17293 |
| Plasmid: pLenti-puro-Flag-MLLS-WT | This paper | N/A |
| Plasmid: pLenti-puro-Flag-MLLS-W141A | This paper | N/A |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead contact**
Lih-Wen Deng (bchdlw@nus.edu.sg)

**Materials availability**
Plasmids generated in this study are available upon request from the lead contact, Lih-Wen Deng (bchdlw@nus.edu.sg), but a completed Materials Transfer Agreement may be required.

**Data and code availability**
Raw sequencing reads have been deposited to GEO: GSE196935. The accession number is also listed in the key resources table. Original western blot images and processed RNA-seq data have been deposited at Mendeley. The DOI is listed in the key resources table.

This paper does not contain original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**
MIIS-KO mouse was generated as previously described (Zhou et al., 2018). Mice were housed in a temperature-controlled room at 24°C with a 12 h light/dark cycle. Fresh water and rodent diet were continuously available. MIIS-WT and MIIS-KO mice used in this study were littermates with same sex on C57BL/6J background at ages indicated in specific experiments. Specifically, ERG was performed with female mice at the age of P16. RNA-seq, qPCR, co-IP and qChIP were performed with P18 female mice retinas, while ISH and immunostaining were performed with P21 male mice retinas. All experimental procedures conformed to the ARVO statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Institutional Animal Care and Use Committee (IACUC) of NUS (Singapore), City University of Hong Kong (Hong Kong, China) and Wenzhou Medical University (Wenzhou, China).
METHOD DETAILS

Cell culture

The murine photoreceptor-derived 661W cell line was a kind gift from Dr. Muayyad R. Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). 661W cells, embryonic kidney cell line HEK293FT, and HEK293T cells were maintained in DMEM containing 10% FBS and 2 mM L-glutamine at 37°C under a humidified atmosphere of 5% CO₂.

siRNA transfection and plasmid constructions

MLL5-specific siRNA duplexes #1 (sense: 5’-TGCTGAGAGAACAGTTTG-3’; anti-sense: 5’-TCAACAGTTTCTCTCAGCA-3’) and #2 (sense: 5’-CAGCCCTCTGCAAACTTTCAGAATT-3’; anti-sense: 5’-AATTCTGAAAGTTTGCAGAGGGCTG-3’) targeting nucleotide position 1,088 and 4,693 respectively from the translation starting point of murine MLL5 (National Center for Biotechnology Information (NCBI) reference sequence: NM_026984.1) were generated from 1st BASE (Singapore). Scrambled siRNA was designed as previously described (Yew et al., 2011). siRNAs were transfected into cells using RNAiMAX Lipofectamine (13667-150; Invitrogen) following the manufacturer’s instructions. MLL5-specific shRNA targeting nucleotide position at 1,556 from the transcription starting point of human MLL5, and scrambled shRNA were cloned into the pLKO.1 puro vector (Addgene #8453) with pLgw-MLL5-V5-EcoDam and pLgw-RFC1-V5-EcoDam, were kindly provided by Dr Bas Van Steensel (Netherlands Cancer Institute). To generate constructs for the luciferase assay, a 1510-bp fragment containing the distal and proximal promoter of human Rho gene (nucleotides -1500 to +10) was cloned into pGL3-basic (Promega) vector with NheI and HindIII sites. Similarly, 1.5-kb upstream genomic fragments of the murine Opn1sw gene (-1450 to -11) were subcloned into the PGL3-basic vector pDONR 221-MLL5 with the lentiviral destination vector pLgw-RFC1-V5-EcoDam using LR Clonase II. DamID lentiviral EcoDam plasmid for the DamID experiments was generated by Gateway recombination of pDONR 221-MLL5 with the lentiviral destination vector pLgw-RFC1-V5-EcoDam using LR Clonase II. DamID lentiviral vectors, pLgw-V5-EcoDam and pLgw-RFC1-V5-EcoDam, were kindly provided by Dr Bas Van Steensel (Netherlands Cancer Institute). To generate constructs for the luciferase assay, a 1510-bp fragment containing the distal and proximal promoter of human Rho gene (nucleotides -1500 to +10) was cloned into pGL3-basic (U47295; Promega) vector with NheI and HindIII sites. Similarly, 1.5-kb upstream genomic fragments of the murine Opn1sw gene (-1450 to +10) were subcloned into the pGL3-basic vector to generate MmOpn1sw-MmOpn1sw luciferase reporter plasmids. All constructs generated by PCR were verified by sequencing. All viral transfer plasmids were amplified in Stbl3 competent cells.

RNA extraction, cDNA synthesis, and quantitative PCR (qPCR)

661W cell pellets or retinas dissected from Mll5-WT or Mll5-KO mice at the indicated ages were homogenized using TRIzol reagent (155967-026; Invitrogen), followed by chloroform-isopropanol-alcohol extraction for total RNA. Two micrograms of total RNA were then reverse transcribed to cDNA using the SuperScript III RT-PCR system (18080-051; Invitrogen). cDNA was diluted and quantified using iTaq universal SYBR green supermix (172-5120; Bio-Rad Laboratories) with an iQ5 Multicolor Real-Time PCR machine (Bio-Rad Laboratories). Primers are listed in Table S2. Relative expression was determined from three independent experiments with three repeats for each experiment and analysed by student t-test.

Immunoprecipitation (IP) and Western blotting

To monitor MLL5 and CRX association by co-IP, retinas dissected from P18 mice were homogenized in a glass homogenizer and lysed in a buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.5% NP-40,
A, 1 mM Na$_3$VO$_4$, 5 mM Mn$_2$O$_4$, and 10 mM β-glycerophosphate). To dissect which domain of Mll5 interacts with CRX, HEK293T cells cultured in 60-mm dishes were sequentially transfected with pXJ40-HA-CRX plasmids (4 μg) and pEF6-Flag-Mll5 (8 μg) or pEF6-Flag-Mll5-PS/-CD/-CT/-CD4/-ΔCD4 (4 μg). Cells were harvested 48 h after transfection and lysed with 1 mL lysis buffer for 15 min on ice. Lysates were then centrifuged at 12,000 g for 10 min, pre-cleared with anti-Mouse/Rabbit Ig IP Beads (Rockland Immunochemicals) for 30 min, and incubated with 2 μg anti-Mll5 antibody, anti-CRX antibody, anti-Flag antibody, or anti-HA antibody for 3 h, followed by a 2 h incubation with 10 μL of anti-Mouse/Rabbit Ig IP Beads. The protein-bound beads were rinsed four times with cell lysis buffer, eluted with 2xLaemmlie sample buffer containing 200 mM DTT, and analysed by western blotting. Luminescent signals were captured using the ChemiDoc™ Imaging System and quantified by ImageJ software. To prepare direct lysates for western blotting, tissues or cells were harvested and rinsed twice with ice-cold PBS, and lysed with RIPA buffer supplied with proteinase inhibitors. Tissues or cells were then sonicated at 20% amplitude on ice with a Vibra-Cell sonicator until they were fully lyzed. The lysates were then incubated on ice for 10 min and centrifuged at 12,000 g for 10 min. The supernatants were then transferred to new tubes and measured protein concentration with Bradford assay, denatured with 2x Laemmlli sample buffer containing 200 mM DTT. An equal amount of protein (10 μg) was used for western blotting analysis.

**Histology and immunohistochemistry**

Whole eyeballs were dissected and fixed in Davidson’s fixative buffer (0.8% PFA, 35% ethanol, and 10% glacial acetic acid) overnight. After rinsing with PBS, eyeballs were subjected to dehydration through increasing concentration of ethanol and xylene, followed by embedding into paraffin. Five-micron paraffin sections were generated on a Leica RM2235 microtome and mounted onto slides. Sections were then rehydrated through decreasing concentration of ethanol (100%, 95%, 70%, and 50%) and subsequently stained with haematoxylin and eosin (H&E) for morphological assessments or used for immunohistochemistry. For immunohistochemistry, sections were sections were permeabilised in 0.5% Triton X-100 in PBS for 15 min after antigen retrieval, and then incubated in blocking buffer (5% BSA in PBS) for 2 h. After incubation with primary antibodies in blocking buffer overnight at 4°C, sections were then incubated with secondary antibodies for 2 h at room temperature (RT). Images were obtained using a confocal fluorescence microscope FV 1000 (Olympus) equipped with a built-in laser scanning unit, using FLU OVI EW viewer software (Ver. 4.2; Olympus).

**Mouse retina in situ hybridization**

In situ hybridization using RNAscope probes was performed following the manufacturer’s protocol for RNAscope 2.5 HD Detection Kit-Brown (ACD, Catalog No. 322310). Briefly, eyeballs dissected from P21 mice were fixed in fresh 10% Neutral buffered formalin for 24 h at RT, dehydrated, and embedded in paraffin. 5μm eyeball sections were baked, deparaffinized and air dry for 5min, followed by incubation in hydrogen peroxide for 10 min at RT. After target retrieval by boiling in target retrieval buffer for 15 min, slides were briefly washed with distilled water and pre-treated by incubation with Protease Plus (ACD, Catalog No. 322331) for 30 min at 40°C. After that, the manufacturer’s protocol for RNAscope 2.5 assay was followed exactly to hybridize probes and detect the signals. RNAscope probe used for Mll5 detection was designed to target nucleotide position 436-1591 of murine Mll5 (ACD, Catalog No. 575421).

**Focal electrotoretinogram (fERG)**

Scotopic and photopic responses in both eyes of an individual mouse were recorded with a well-established Ganzfeld dome stimulating and data collecting system (Q450SC UV; Roland, Wiesbaden, Germany). Briefly, mice were dark-adapted for 6 h and subsequently anaesthetised with an intraperitoneal injection of a mixture of ketamine (72 mg/kg) and xylazine (4 mg/kg). Pupils were then dilated with 1% atropine and 2.5% phenylephrine hydrochloride before the experiment. Ground electrodes and a referential needle were punctured into the tail and cheek, respectively. The mice were then stimulated with five levels of stimuli ranging from 3.0 to -1.7 log cd·s/m² to elicit scotopic ERGs. After 10 min of light adaption with a background light of 5 log cd·s/m², the mice were then subjected to photopic ERG measurement upon stimulation with five levels of stimuli ranging from 0.7 to 3.3 log cd·s/m².

**RNA-seq**

For the preparation of total RNA for RNA-seq, 8 eyeballs of two pairs of female Mll5-WT and Mll5-KO mice at the age of P18 were dissected. Total RNA was extracted with RNasy mini kit (Qiagen, 74104).
A minimum of 2 μg of total RNA obtained from mixture of 2 eyeballs were used for library construction, and the remaining RNA were used for cDNA synthesis and qPCR analysis. Library sequencing was performed on the Illumina HiSeq-PE150 platform. RNA-seq results from two experimental repeats were presented.

**Virus production and transduction**

Lentivirus production was performed as previously described (Zhao et al., 2016) with the following modifications. HEK293FT cells cultured in 60-mm dishes were co-transfected with lentiviral transfer constructs (pLenti-puro-Flag-MLL5-WT/-W141A/-T440A, pLenti-puro-empty, pLgw-MLL5-V5-EcoDam, or pLgw-V5-EcoDam; 4 μg), packaging construct pCMV-ΔR8.91 (encoding the HIV-1 gag-pol; 2 μg), along with PMD2.G (encoding the VSV-G envelope; 0.4 μg) using the calcium phosphate method. The lentivirus-containing supernatant was harvested on three consecutive days and concentrated. For the production of retrovirus for 661W transduction, the procedure above was performed, except that transfection plasmids were pMSCVneo-HA-CRX-IRES-GFP or pMSCVneo-empty (4 μg), together with PCL-Eco (2.0 μg), VSV-G (1.0 μg), and Gag-Pol plasmids (1.0 μg). After transfection, the virus-containing cell supernatant was diluted in DMEM complete medium in the presence of 8 μg/mL polybrene and added to 661W cells. After 24 h incubation, medium was replaced with fresh culture medium and incubated for a further 48 h. Cells were then subjected to selection using 2.5 μg/mL puromycin or 2 mg/mL G418 selection for 24 h before harvesting.

**Dual-luciferase assay**

To measure the transcription activity of HsRho, MmOpn1mw, and MmOpn1sw promoters, HEK293T cells in 24-well plates were transfected with pEF6-Flag-MLL5 or pEF6-Flag empty plasmid (0.6 μg), followed by a subsequent transfection with pGL3-Rho promoter-Luc plasmids (0.4 μg) together with pXJ40-HA-CRX DNA (0.4 μg) and pRL-TK-Renilla luciferase expression vector (0.1 μg) using a standard calcium phosphate method. Depletion of endogenous MLL5 expression in HEK293T cells was achieved by transfection of shRNA duplexes, using the calcium phosphate method. Each plasmid combination was transfected in duplicate. Cells were harvested 48 h post-transfection for western blotting and luciferase assays. Relative luminometer units (RLU) result from Firefly luciferase reaction and Renilla luciferase reaction were measured with a Dual-luciferase assay system (E1910; Promega) on a Synergy H1 microplate reader (BioTek). Each firefly luciferase RLU was normalised to the control Renilla luciferase RLU, and was presented as relative fold change compared to negative controls.

**Chromatin immunoprecipitation (ChIP) analysis**

An EpiQuik™ chromatin immunoprecipitation kit (P-2002) from Epigentek Group Inc. (Brooklyn, NY) was used following the manufacturer’s protocol. Briefly, P18 mouse retinas were dissected in ice-cold PBS prior to homogenisation and crosslinking. Chromatin was sonicated to obtain sheared DNA fragments of 200-1000-bp in length. One microgram of antibody was used per immunoprecipitation (IP) from approximately 2 million retinal cells or 661W (CRX OE) cells. DNA from precipitated chromatin and input controls were analysed by qPCR using specific primers for the promoter or 3′ UTR regions of the Rho gene, as well as primers for Collagenase gene promoter. A pair of primers recognising a region of chromosome 8 that does not contain any histone modifications was included as a negative control. All qPCR primer sequences are described in Table S2. The difference was calculated as: % Input = 2^[(Input Ct – (log x)/(log2)-ChIP Ct)] *100%, where “x” is the ratio of ChIP sample volume to Input sample volume.

**DamID assay**

DamID was performed as previously described (Vogel et al., 2007). Briefly, 661W (EV OE) and 661W (CRX OE) cells were incubated overnight with lentivirus expressing MLL5-Dam or Dam-control proteins and then cultured for another 48 h before subjected to selection using 2 mg/mL G418 for 24 h. Cells were then subjected to a standard DamID protocol (Vogel et al., 2006). Briefly, genomic DNA was extracted using the DNeasy Tissue kit (Qiagen). Genomic DNA (2.5 μg) was digested with DpnI and ligated to adaptors, followed by DpnII digestion. GATC methylated DNA was then amplified by ligation-mediated PCR with Advantage cDNA polymerase mix (639105; Clontech). Amplicons were purified and subjected to quantitative PCR analysis with gene-specific primers (Table S2).
QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using GraphPad Prism 6.01. Significance levels were obtained by student t test and indicated by *p < 0.05, **p < 0.01, ***p < 0.001, ns (not significant when p ≥ 0.05). Error bars represent the SEM of three independent experiments.