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The Transcription Factor Neural Retina Leucine Zipper (NRL) Controls Photoreceptor-specific Expression of Myocyte Enhancer Factor *Mef2c* from an Alternative Promoter*

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Background: *Mef2c* gene expression is significantly diminished in the retinas of NRL (neural retina leucine zipper) knock-out mice.
Results: NRL binding, RNA polymerase II association, and acetylation of histone H3, revealed a novel alternate *Mef2c* promoter.
Conclusions: Activation of the retinal *Mef2c* promoter is NRL-dependent and specific to rod photoreceptor cells.
Significance: *Mef2c* represents a novel regulatory node downstream of NRL in adult rod photoreceptor cells.

Neural retina leucine zipper (NRL) is an essential transcription factor for cell fate specification and functional maintenance of rod photoreceptors in the mammalian retina. In the $Nrl^{-/-}$ mouse retina, photoreceptor precursors fail to produce rods and generate functional cone photoreceptors that predominantly express S-opsin. Previous global expression analysis using microarrays revealed dramatically reduced expression of myocyte enhancer factor *Mef2c* in the adult $Nrl^{-/-}$ retina. We undertook this study to examine the biological relevance of *Mef2c* expression in retinal rod photoreceptors. Bioinformatics analysis, rapid analysis of cDNA ends (5′-RACE), and reverse transcription coupled with qPCR using splice site-specific oligonucleotides suggested that *Mef2c* is expressed in the mature retina from an alternative promoter. Chromatin immunoprecipitation (ChIP) studies showed the association of active RNA polymerase II and acetylated histone H3 just upstream of *Mef2c* exon 4, providing additional evidence for the utilization of an alternative promoter in the retina. In concordance, we observed the binding of NRL to a putative NRL-response element (NRE) at this location by ChIP-seq and electrophoretic mobility shift assays. NRL also activated the *Mef2c* alternative promoter *in vitro* and *in vivo*. Notably, MEF2C could support *Rhodopsin* promoter activity in rod photoreceptors. We conclude that *Mef2c* expression from an alternative promoter in the retina is regulated by NRL. Our studies also implicate MEF2C as a transcriptional regulator of homeostasis in rod photoreceptor cells.

Distinct gene expression patterns dictate cellular identity and function (1–3). Spatiotemporal control of gene expression is achieved by integrating multiple regulatory mechanisms, including transient or stable interactions between *cis*-regulatory DNA elements and *trans*-regulatory factors, epigenetic code, and signaling molecules (4). The neural retina provides an excellent paradigm to elucidate qualitative and quantitative control of gene expression in the developing and adult central nervous system (5). In the retina, six types of neuronal cells and one type of glial cell (Muller) differentiate from multipotent progenitor cells in a conserved order of birth, with each key step controlled by transcriptional regulatory proteins (6, 7). Rod and cone photoreceptors in the retina are light sensors that convert photons into electrical signals (phototransduction) under dim and bright light conditions, respectively (8). Cell type-specific genes are activated during the differentiation and maturation of photoreceptors (5). Precise control of gene expression is also essential for photoreceptor survival as both under- or overexpression of *Rhodopsin* and other genes can lead to photoreceptor degeneration (9–11). Dysfunction and death of rod photoreceptors is a common hallmark of many inherited retinal dystrophies in humans (5, 12). Pathological mutations involve genes required for phototransduction, outer segment morphogenesis, intracellular transport, and/or transcription factors that control the expression of genes involved in photoreceptor homeostasis (12).

Photoreceptor development and homeostasis are tightly regulated by key transcription factors that include orthodenticle homeobox 2 (13), cone rod homeobox (CRX)[2] (14–17), photoreceptor-specific orphan nuclear receptor (NR2E3) (18–21), thyroid hormone receptor β2 (TRβ2) (22), retinoid-related orphan receptor β (RORβ) (23, 24), neural retina leucine zipper (NRL) (17, 25, 26), and estrogen-related receptor β (27). Although several of these transcriptional regulators are individually essential to generate a normal compliment of rod and cone photoreceptors during development, they also function

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[2] The abbreviations used are: CRX, cone rod homeobox; Nrl, neural retina leucine zipper; qPCR, quantitative polymerase chain reaction; Mef2c, myocyte enhancer factor-2c; RACE, rapid analysis of cDNA ends; ChIP-seq, ChIP followed by high throughput sequencing; ChIP-on-chip, ChIP followed by tiling array analysis; NRE, NRL response element; P, postnatal day; Pol II, polymerase II; TdT, Td tomato.

cooperatively to regulate photoreceptor-specific genes in the mature retina (5).

NRL is the primary regulator of rod *versus* cone photoreceptor cell fate choice (25). Abrogation of *Nrl* expression in the mouse leads to a complete loss of rod photoreceptors and an increase in the fraction of cones expressing S-opsin (25). Ectopic expression of *Nrl* results in a rod-only mouse retina devoid of cone photoreceptors (28). NRL and its interacting protein CRX activate many rod photoreceptor-specific genes, whereas NRL and its downstream transcriptional target NR2E3 repress cone genes (18, 20, 21, 26, 29). Mutations of human *NRL* cause retinal degenerative diseases (30–32).

Nrl expression in mice is detected as early as embryonic day 12.5; however, the expression of phototransduction genes including *Rhodopsin,* are not detected until age P3 (5, 33). This "delay" in expression of rod maturation genes suggests the involvement of additional regulatory signals. We previously showed that NR2E3 is a direct transcriptional target of NRL (26). The primary role of NR2E3 appears to be suppression of cone gene expression, although it can contribute to the induction of rod-specific genes (18, 20, 21, 34). Estrogen-related receptor β, another potential target of NRL, participates in rod survival by regulating the expression of many genes (27). To identify additional transcription factors that control rod-specific gene expression, we took advantage of gene profiles generated from wild type and $Nrl^{-/-}$ retina and from their respective purified photoreceptors (33, 35). A survey of genes that are down-regulated in the absence of *Nrl* identified *Mef2c* (myocyte enhancer factor 2c) as a potential novel target of NRL.

MEF2C belongs to the MADS (MCM1-agamous-deficiens serum response factor) family of transcription factors and is essential for muscle, cardiovascular, and bone development (36–38). Among the four vertebrate MEF2 proteins, postnatal expression of MEF2C is restricted to muscle, brain, and spleen, whereas others (MEF2A, MEF2B, and MEF2D) are expressed ubiquitously (39). MEF2C and two other transcription factors (GATA4 and TBX5) are sufficient to reprogram mouse fibroblasts into functional cardiomyocytes (40). Conditional knockout of *Mef2c* in mouse brain has revealed its essential role in early neurogenesis, neuronal migration, and differentiation (41, 42). MEF2C activity is modulated by post-translational modifications in response to cytoplasmic signals including calcium (37). Mutations in human *MEF2C* are associated with neurological disorders including mental retardation and seizures (43, 44).

To examine the expression and function of *Mef2c* in the retina, we have performed a comprehensive analysis using *in silico*, *in vitro*, and *in vivo* methods. Here we show that *Mef2c* transcripts in the retina originate from an alternative promoter upstream of exon 4. We also demonstrate that the *Mef2c* promoter is a direct transcriptional target of NRL, and MEF2C contributes to the regulation of *Rhodopsin* promoter activity *in vivo*.

EXPERIMENTAL PROCEDURES

Animal Care and Use—Animal Care and Use committees of Oakland University and the National Eye Institute approved all animal care and tissue collection procedures. C57BL/6J, rd1, and CD1 mice were obtained from Charles River Laboratories (Wilmington, MA) or the Jackson Laboratory (Bar Harbor, ME).

Antibodies—The following ChIP grade antibodies were used: anti-Pol II, a polyclonal antibody to total RNA polymerase II (Santa Cruz Biotechnology, Santa Cruz, CA); anti-Pol-II-S2, an antibody against transcriptionally active Pol II, phosphorylated on serine 2 of the C-terminal repeat domain (Abcam Inc., Cambridge, MA); anti-H3K9-Ac, a polyclonal antibody to histone H3 acetylated on lysine 9 (Abcam Inc.); and anti-NRL polyclonal antibody (45).

Cloning of the Mouse Mef2c Retinal Proximal Promoter and shRNA Plasmids—A 540-bp genomic fragment around *Mef2c* exon 4 was amplified from C57BL/6 mouse genomic DNA using *Pfu* Ultra (Agilent Technologies, Santa Clara, CA) and cloned into pGemTeasy (Promega, Madison, WI) to produce pGemTeasy-*Mef2c*P-Ret. The PCR primers including restriction sites for KpnI and XhoI are listed here with the genomic sequences underlined: forward KpnI, 5′-CGCAGGTAC-CTTTCATGTGTGTTCATATTTGCAC-3′, reverse XhoI, 5′-TGCACTCGAGATACCCCAATGAGACAAGAAGGC-3′. The *Mef2c* promoter was subcloned from pGemTEasy-*Mef2c*P-Ret into the KpnI/XhoI sites of pGL3-basic (Promega) to produce the luciferase-reporter construct: pGL3-*Mef2c*P-Luc. The same *Mef2c* promoter was released from pGL3-*Mef2c*P-Luc using KpnI/XhoI, blunt ended, and ligated into a GFP vector to produce the *Mef2c*P-GFP construct. Sequencing validated all constructs. The *Nrl* shRNA plasmid was generated to knockdown *Nrl* expression (46). *Mef2c* and *Gapdh* shRNA plasmids were purchased from Open Biosystems (Huntsville, AL).

5′ Rapid Analysis of cDNA Ends Derived from Full-length RNA (RACE)—Total RNA was isolated from mouse skeletal muscle, brain, and retina, respectively, using TRIzol reagent (Invitrogen). 5′-RACE was performed using the GeneRacer™ kit (Invitrogen) according to the manufacturer's instructions (47, 48). Briefly, 2 μg of RNA was treated with calf intestinal phosphatase to eliminate truncated mRNA and non-mRNA. End caps were removed from full-length mRNAs with tobacco acid pyrophosphatase, leaving a 5′-phosphate for ligation to the GeneRacer RNA oligo. Reverse transcription with random hexamers was used to synthesize cDNA. To obtain 5′ ends, the first strand cDNA was amplified using a Mef2c-specific reverse primer (5′-ATCTCACAGTCGCACAGCAC-3′) and the GeneRacer™ 5′ primer. The RACE PCR product was visualized on a 1.5% agarose gel, purified, and cloned into the pCR 4-TOPO vector using the TOPO TA cloning kit (Invitrogen) for sequencing. At least 15 clones were sequenced for each reaction.

RNA Polymerase II (Pol II) Chromatin Immunoprecipitation (ChIP) Hybridized to Promoter Tiling Arrays (ChIP-on-chip)—Data for the *Mef2c* gene were derived from the complete Pol II ChIP-on-Chip data set, which is available under GEO accession number GSE 19999. ChIP-on-chip was performed as previously detailed (49). Briefly, Pol II ChIP was carried out using retinas from CL57BL/6 mice at age P2 and P25. The ChIP DNA was amplified in a linear fashion using random priming amplification, fragmented, and labeled using the GeneChip Double-stranded DNA

Terminal Labeling Kit (Affymetrix, Santa Clara, CA). The resulting biotinylated DNA probe was hybridized to GeneChip Mouse Promoter 1.0R arrays (Affymetrix). Biotinylated probe prepared from total genomic DNA (non-ChIP) was hybridized to a separate GeneChip for paired normalization. Tiling arrays were scanned with an Affymetrix GeneChip scanner. Fluorescence data for the ChIP samples (P2 and P25) were normalized to the total genomic DNA control using Affymetrix Tiling Analysis software. Signal values were exported in bar-file format for interval determination with Affymetrix Tiling Analysis software and visualization with Integrated Genome Browser software. Track intervals parameters were: minimum length 300, gap 180, and intermediate threshold of 4.

ChIP Quantitative Real-time PCR (qPCR)—ChIP assays using an antibody recognizing the active form of Pol II (50), or H3K9-Ac were performed with P2 and P25 mouse retinas as previously described (51). The ChIP DNA was quantified in triplicate by real time qPCR using SYBR Green Super mixture (Bio-Rad). The copy number of ChIP DNA for each test region was obtained by comparing the C_t values from ChIP DNA with a standard curve composed of known copy numbers of genomic DNA and their corresponding C_t values. The ChIP-qPCR signal was normalized to the qPCR signal from input DNA, and calculated as copies of DNA detected per 1000 genome equivalents of input DNA. An untranslated region on Chromosome-6 (Untr) served as a negative control (49). Primer sequences used were: *Mef2c* exon 4 promoter (forward, 5′-TGCAGAAAAGATTCCCACTTG-3′, reverse, 5′-AGACACTCACAAGGCAAAGAC-3′), *Rhodopsin* promoter (forward, 5′-CCCCTCTGCAAGCCAATT-3′, reverse, 5′-GCAACTCCAGGCACTGAC-3′), *Recoverin* (*Rcvrn*) promoter (forward, 5′-CTCCTCCCTCCAAGGACTG-3′, reverse, 5′-CAAGGCTGTGTGCTGCTATG-3′), Untr (forward, 5′-TCAGGCATGAACCACCATAC-3′, reverse, 5′-AACATCCACACGTCCAGTGA-3′).

NRL ChIP-sequencing (Seq) and ChIP Assays—ChIP using NRL antibody or normal IgG control was performed as described (26). ChIP DNA from retinas of C57BL/6J mice (age P28), was used to construct NRL and IgG (control) ChIP-seq libraries, according to the manufacturer's protocol (Illumina, San Diego, CA). Briefly, ChIP DNA was end-repaired, ligated to universal adaptors, and amplified in a linear fashion. The amplified DNA was purified and used for cluster generation and sequencing analysis using the Illumina 1G Genome Analyzer. The ChIP-seq reads were mapped to the mouse genome (mm8) using the Solexa Analysis Pipeline and NRL-binding regions were identified by Model-based Analysis of ChIP-seq (MACS) with a false discovery rate $<10^{-6}$ (52).

For ChIP assays, separate experiments were performed using retinas from C57BL/6J mice and $Nrl^{-/-}$ mice (on C57BL/6J background) at P2 and P28 using NRL antibody. Normal rabbit IgG was used as a negative control. The ChIP DNA and input DNA control (without immunoprecipitation) were analyzed by PCR using the following primers: *Mef2c* exon 4 promoter (forward, 5′-TGCAGAAAAGATTCCCACTTG-3′, reverse, 5′-AGACACTCACAAGGCAAAGAC-3′), *Rhodopsin* promoter (forward, 5′-CCCCTCTGCAAGCCAATT-3′, reverse, 5′-GCAACTCCAGGCACTGAC-3′), *Rhodopsin* intron negative control (forward, 5′-TGTGGTCTTCACCTGGATCATG-3′, reverse, 5′-TACCTGGACCAGCCAACGA-3′).

Gene Expression Assays—Total RNA was isolated from C57BL/6 or Rd1 mouse retinas using the Absolute RNA Miniprep kit, and cDNA was synthesized using the AffinityScript qPCR cDNA Synthesis kit (Agilent Technologies, La Jolla, CA). Gene expression was detected by qPCR using TaqMan probes and 2× Gene Expression Master mix (Applied Biosystems, Foster City, CA) on an MX3000P real time PCR unit (Agilent).

Cell Culture, Transient Transfection, and Dual Luciferase Assay—HEK293 cells (American Type Culture Collection, Manassas, VA) were cultured in minimal essential medium-α containing 10% FBS and 100 units/ml of penicillin/streptomycin at 37 °C with 5% CO_2. The cells were seeded into 24-well plates (150,000 cells/well), 24 h before transfection with Lipofectamine 2000 (Invitrogen) as described previously (17, 53). Triplicate wells were co-transfected with the Firefly luciferase reporter construct, pGL3-*Mef2c*P-Luc (667 ng/ml); the transfection control plasmid, *Renilla* luciferase reporter pRL-CMV (11 ng/ml); and expression plasmid for human NRL (pED-NRL at 55 or 278 ng/ml) and/or expression plasmid for human CRX (pcDNA3.1/HisC-hCRX at 278 ng/ml). The corresponding empty vectors (pED, pcDNA3.1/HisC) were used to adjust the total amount of transfected DNA. Cells were harvested 48 h after transfection for assay of Firefly and *Renilla* luciferase activities using the Dual Reporter Luminescence Reagent and a Turner dual-injector luminometer (Promega). *Renilla* luciferase activity was used to normalize for transfection efficiency. Fold-activation was calculated relative to transfection with empty expression vectors. All experiments were repeated at least three times. Statistical comparisons used analysis of variance with Tukey-HSD post-analysis using VassarStats.

Electrophoretic Mobility Shift Assay (EMSA)—EMSA was performed as previously described (26). Briefly, nuclear extracts from transfected HEK 293T cells were prepared using a kit (Active Motif, Carlsbad, CA). The DNA oligonucleotide (NRE, forward, 5′-TAGACAGTGACCTCCTCCCTGCTGAGCCACTATGCTCT-3′, NRE reverse, 5′-AGAGCATAGTGGCTCAGCAGGGAGGAGGTCACTGTCTA-3′) contain a putative NRL-response element (NRE) (underlined) predicted by Genomatix programs in the mouse *Mef2c* promoter region. ^{32}P-Labeled double-stranded oligonucleotide (40,000 cpm) was incubated with nuclear extracts at 4 °C for 20 min. In competition studies, nuclear extracts were preincubated with a 50-fold excess of unlabeled oligonucleotide for 30 min at room temperature and incubated with labeled oligonucleotide for 20 min. A mutant oligonucleotide (forward, 5′-TAGACAGTGACCTCCTCCCTGCCGGGTCGCTATGCTCT-3′, reverse, 5′-AGAGCATAGCGACCCGGCAGGGAGGAGGTCACTGTCTA-3′) with four nucleotides changed in the NRE site was also used to compete for the protein binding to the oligonucleotide. To test the presence of NRL in the protein-DNA complexes, nuclear extracts were incubated with 2 μg of the anti-NRL antibody or normal rabbit IgG for 30 min at room temperature, followed by the addition of labeled oligonucleotide and a further incubation for 20 min at room temperature. The reaction mixtures were electrophoresed on 8% polyacrylamide gels at 100 V for 1.5 h and subjected to autoradiography.

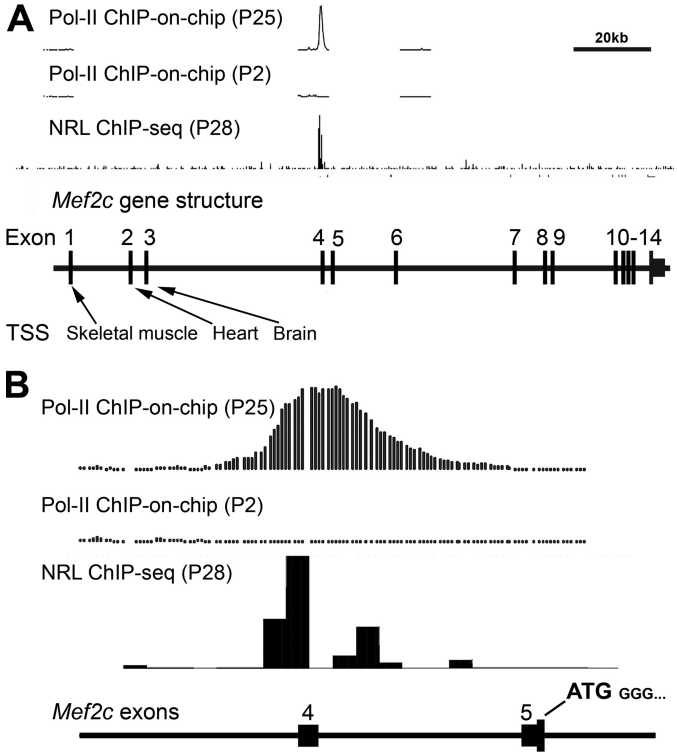

FIGURE 1. **Identification of a novel retina-specific promoter of *Mef2c* by ChIP-on-chip and ChIP-seq analysis.** *A*, RNA Pol II ChIP-on-chip peaks at P25 and P2, and NRL ChIP-seq peaks at P28 were mapped to the mouse genome. The *Mef2c* gene structure is aligned in scale with the exons (*vertical black bars*) numbered. Tissue-specific *Mef2c* transcription start sites (*TSS*) previously reported for skeletal muscle, heart, and brain are indicated. *Scale bar,* 20 kb. *B*, Pol II ChIP-on-chip peaks (P25 and P2) and NRL ChIP-seq peaks (P28) at the *Mef2c* locus plotted at higher resolution. The *Mef2c* start codon (ATG) is located in exon 5, and is common to all known tissue-specific transcripts.

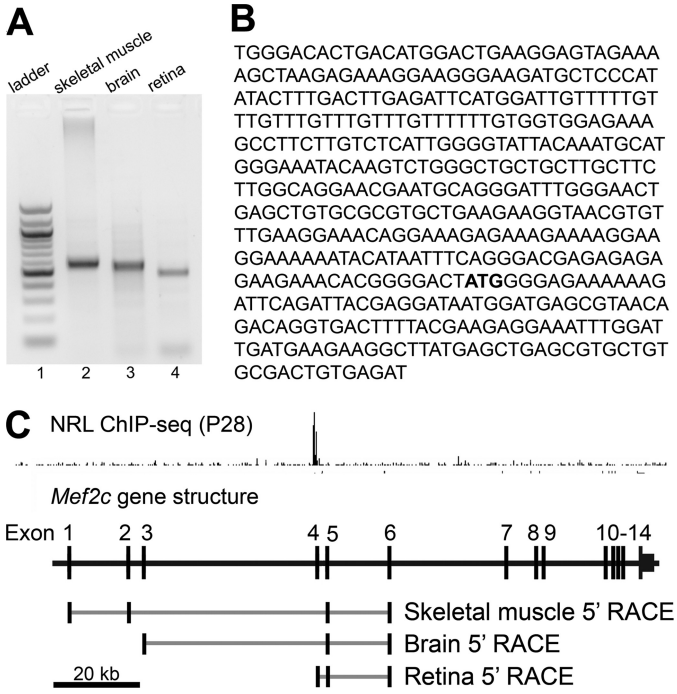

FIGURE 2. **Mapping of retinal *Mef2c* promoter by 5′-RACE.** The 5′-untranslated regions of the *Mef2c* gene in mouse skeletal muscle, brain, and retina were defined by 5′-RACE. The 5′-RACE products were visualized on a 1.5% agarose gel (*A*), purified, and cloned for sequencing (*B*). The 5′ UTR sequences were aligned to the *Mef2c* gene structure (*C*). At least 15 clones were sequenced for each reaction. *A*, DNA ladder (*lane 1*) and tissue-specific 5′-RACE products using RNA from mouse skeletal muscle (*lane 2*), brain (*lane 3*), and retina (*lane 4*) were visualized on a 1.5% agarose gel. *B*, the retina-specific 5′ UTR sequence in which the *Mef2c* start codon (ATG) is shown in *bold*. *C*, alignment of the 5′-RACE sequences to the *Mef2c* gene structure. Exons are shown as *black bars* and the spliced regions in *gray lines*. NRL ChIP-seq peaks at P28 were mapped to the mouse genome. The *Mef2c* structure is aligned and shown in scale *under* the peaks. *Scale bar,* 20 kb.

Western Blot—HEK293 cells (300,000 cells) were co-transfected with 0.5 μg of mouse *Mef2c* expression plasmid (Mef2c cDNA), and 0.5 μg of *Gapdh* shRNA or *Mef2c* shRNA 1 or *Mef2c* shRNA 2. Cells were harvested 48 h after transfection and lysed by sonication in radioimmunoprecipitation buffer supplemented with 20 mM *N*-ethylmaleimide and protease inhibitor mixture (Roche Applied Science). The protein concentration of the supernatant was measured by the bicinchoninic acid assay (Thermo Scientific, Waltham, MA) and an equal amount of lysate was boiled in 2× SDS-PAGE loading buffer (Invitrogen). The lysate was resolved by SDS-PAGE and transferred to nitrocellulose membrane (Invitrogen). The membrane was probed sequentially with MEF2C and tubulin antibodies and visualized by enhanced chemiluminescence (Thermo Scientific).

In Vivo Electroporation—Retinas of CD1, C57BL/6J, or $Nrl^{-/-}$ mouse pups at P0 were electroporated *in vivo* as previously described (46, 54, 55). Briefly, equal amounts of plasmids, *Mef2c*P-GFP and CAG-mCherry, were mixed with either *Nrl* shRNA or *Gapdh* shRNA control and injected into the subretinal space of CD1 pups at P0. Equal amounts of *Mef2c*P-GFP and CAG-mCherry were injected into the subretinal space of C57BL/6J and $Nrl^{-/-}$ P0 pups. Equal amounts of Rho-TdT (Td tomato) and Ub-GFP were mixed with either *Mef2c* shRNA or *Gapdh* shRNA and injected into the subretinal space of CD1 pups at P0. The injection volume was 0.2 μl. The DNA concentration was 300 nM for all constructs: *Mef2c*P-GFP, CAG-mCherry, Rho-TdT, Ub-GFP, *Nrl* shRNA, *Mef2c* shRNA, and *Gapdh* shRNA. Voltage pulses (80 V, 1 Hz, 5 pulses) were applied across the heads of pups using an ECM830 squarewave electroporator and 10-mm diameter BTX Tweezertrode electrodes (Holliston, MA). Retinas were harvested at P20 and P28. Tissues were fixed in 4% paraformaldehyde, and cryoprotected in 30% sucrose. Cryosections (10 μm) were counterstained with DAPI, and then imaged with an Olympus FluoView FV1000 confocal laser scanner.

RESULTS

Prediction of an Alternative Promoter Regulating Mef2c Expression in the Retina—Global expression profiling of adult $Nrl^{-/-}$ retina or purified photoreceptors demonstrated massive down-regulation of Mef2c transcripts, leading to the hypothesis that *Mef2c* is a transcriptional target of NRL and participates in rod photoreceptor homeostasis (33, 35). The analysis of expressed sequence tags (GenBank™) and previous studies (56) demonstrated that the mouse *Mef2c* gene consists of 14 exons with alternative transcription start sites and alternate splicing in specific tissues. Exons 1, 2, or 3 of *Mef2c* are spliced directly to exon 5 in skeletal muscle, heart, or brain, respectively. These tissue-specific transcripts all include exon 5, which contains the translation start site (56). Our retinal

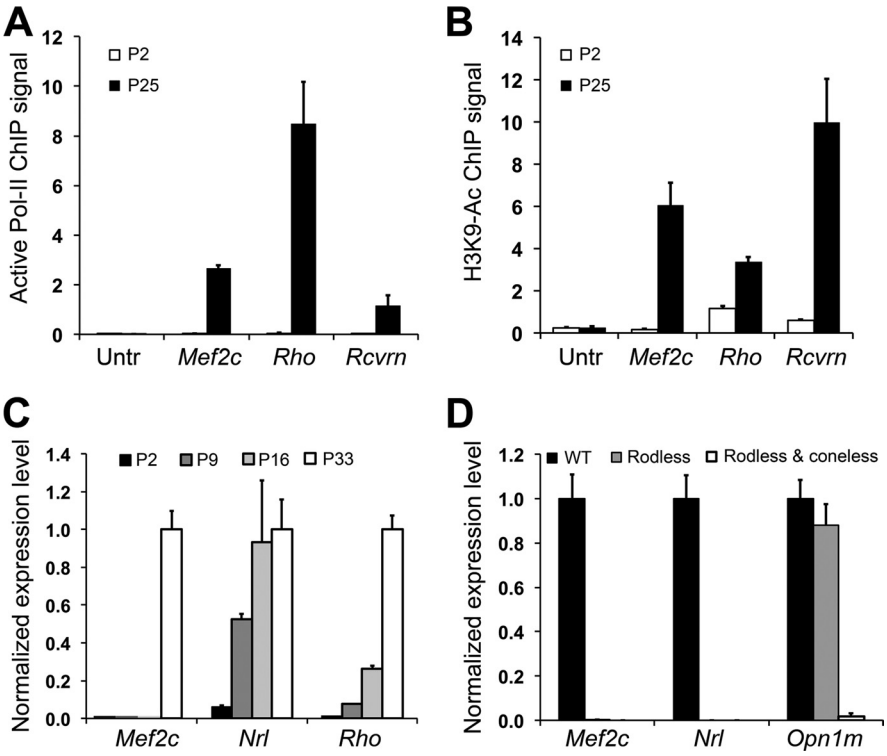

FIGURE 3. **Activation of an alternative *Mef2c* promoter during photoreceptor maturation.** Binding of RNA Pol II and acetylation of histones at the retinal *Mef2c* promoter were measured by ChIP-qPCR using a Pol-II-S2 antibody recognizing the active form of Pol II (*A*) or H3K9-Ac antibody (*B*) in P2 (immature) and P25 (mature) mouse retina. The ChIP DNA was quantified in triplicate by qPCR. Results are presented as copy number detected per 1000 genome equivalents of input DNA. An untranslated region on chromosome-6 (untranslated region, *Untr*) served as a negative control region. *Rhodopsin* (*Rho*) and *Recoverin* (*Rcvrn*) promoter regions served as positive controls. *C*, expression of *Mef2c*, *Nrl*, and *Rhodopsin* were measured by qRT-PCR during retina development (at P2, P9, P16, and P33) and normalized to *β-Actin* (*Actb*). TaqMan probes targeting *Mef2c* exons 5 and 6 were used to detect *Mef2c* expression. *D*, expression of *Mef2c*, *Nrl*, and *M-Opsin* (*Opn1m*) in P33 normal retina (*WT*), P33 rod-less *Rd1* retinas (*rodless*), and P117 *Rd1* retinas lacking both rods and cone (*rodless and coneless*) were measured by RT-qPCR and normalized to *β-Actin* (*Actb*). *Bars* indicate S.D. ($n = 3$).

ChIP-on-chip analysis detected a clear association of RNA Pol II immediately upstream of exon 4, but not upstream of exon 1 (Fig. 1, *A* and *B*), suggesting that most adult retinal transcripts of *Mef2c* start at exon 4. ChIP-seq analysis also detected a strong association of NRL with the genomic region immediately upstream of exon 4, providing further evidence for an alternative promoter that dictates *Mef2c* expression in the neural retina (Fig. 1, *A* and *B*).

5′-RACE Confirms a Retinal Transcription Start Site at Exon 4—5′-RACE PCR products derived from full-length RNA of different tissues (retina, brain, and skeletal muscle) were of different size, demonstrating alternative transcription start sites of *Mef2c* in retina, brain, and skeletal muscle (Fig. 2*A*). Sequence analyses of 5′-RACE products from the neural retina were comprised of sequences aligning to exons 4, 5, and 6 (Fig. 2*B*). Alignment of the tissue-specific 5′-RACE sequences revealed the following preferred transcription start sites: exon 1 for skeletal muscle, exon 3 for brain, and exon 4 for neural retina (Fig. 2*C*). All transcripts included exon 5, which contains the translation start site for the MEF2C protein.

Enhanced Pol II and H3K9-Ac Association with the Novel Mef2c Promoter in Mature Retina Compared with Developing Retina—It should be noted that Pol II ChIP-on-chip only detected significant Pol II association around exon 4 in P25 but not in P2 retina (Fig. 1*A*). We next observed that the transcriptionally active form of Pol II, phosphorylated on serine 2 (Pol-II-S2), associated with the retinal *Mef2c* promoter (exon 4) at age P25 but not at age P2 (Fig. 3*A*). In concordance, ChIP-qPCR analysis also detected Pol-II-S2 association with the promoters of two rod-specific genes, *Rhodopsin* (*Rho*) and *Recoverin* (*Rcvrn*), when the genes were active at P25 but not at P2 (Fig. 3*A*). An intergenic untranslated region (Untr), downstream of *Rho*, was negative for Pol-II-S2 binding at both P2 and P25 (Fig. 3*A*).

Acetylation of lysine 9 on histone H3 favors the formation of an open chromatin architecture and marks actively transcribed genes (57). To examine the epigenetic state of the *Mef2c* promoter, we performed H3K9-Ac ChIP-qPCR and detected increased acetylation of H3K9 corresponding to increased Pol-II-S2 binding at P25 compared with P2 (Fig. 3*B*). We identified a similar increase in H3K9-Ac near the transcription start sites of the two NRL-activated rod-specific genes: *Rho* and *Rcvrn*. H3K9-Ac levels remained at background in the untranslated control region (UTR) at both P25 and P2 (Fig. 3*B*).

Mef2c Is Expressed in Mature But Not in Developing Retinas—We next examined the temporal expression profile of *Mef2c* using a qPCR assay (TaqMan) targeting the exon 5–6 splicing junction in wild type (WT) retina at critical developmental stages: P2, P9, P16, and P33 (Fig. 3*C*). *Mef2c* transcripts were only detected in mature retinas (P33) but not in developing retinas (P2, P9, and P16) (Fig. 3*C*). TaqMan probes specifically targeting *Mef2c* exons 1 and 2 detected relatively little or no transcript in the retina at any developmental stage, compared with exon 5–6 transcripts (data not shown). As predicted, *Nrl*

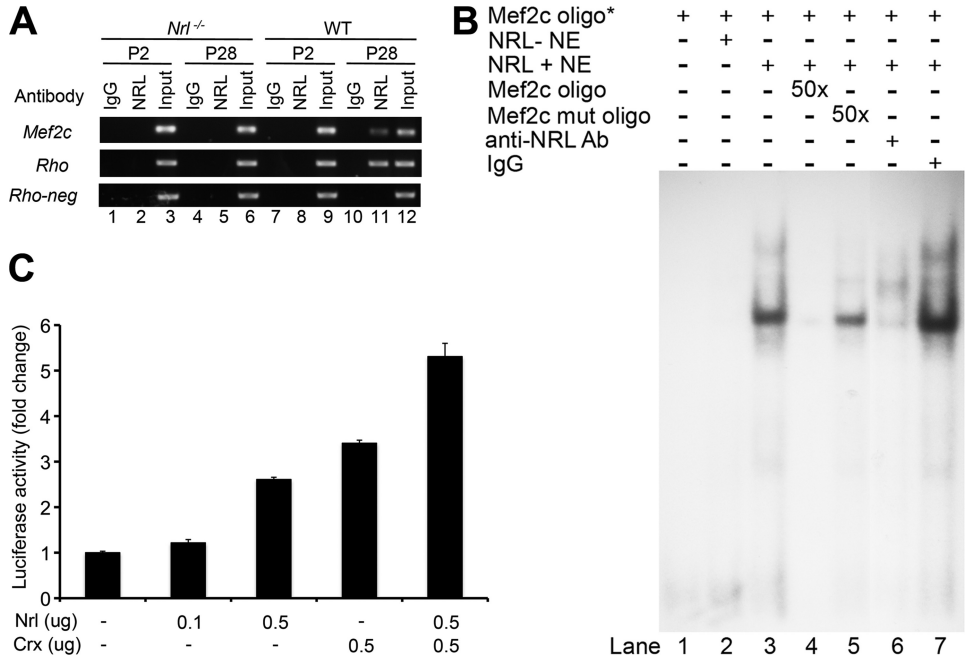

FIGURE 4. **NRL binds to and activates the retinal *Mef2c* promoter.** *A, in vivo* binding of NRL with the retinal *Mef2c* promoter was detected by ChIP assays. ChIP was performed using NRL-deficient ($Nrl^{-/-}$) retina (*lanes 1–6*) and wild type (*WT*) retina (*lanes 7–12*), at P2 (*lanes 1–3* and *7–9*) and P28 (*lanes 4–6* and *10–12*) with antibody to NRL (*lanes 2*, *5*, *8*, and *11*). *Input* (chromatin samples without IP) served as positive control (*lanes 3*, *6*, *9*, and *12*) and IP with normal IgG (IgG) served as negative control (*lanes 1*, *4*, *7*, and *10*). The *Rhodopsin* (*Rho*) promoter was a positive control for NRL binding, whereas a region in *Rho* intron (Rho-neg) served as a negative control. *B*, NRL directly binds to NRE in the *Mef2c* promoter *in vitro*. Components present in each binding reaction are indicated *above* each lane in the autoradiograph. *Mef2c* oligo*, representing a ^{32}P-labeled NRE containing oligonucleotide, was included in all reactions (*lanes 1–7*). Reactions included 10 μg of nuclear extract from HEK293T cells transfected with either empty vector (NRL-NE, *lane 2*) or *Nrl* cDNA expression plasmid (NRL+NE, *lanes 3–7*). *Lanes 4* and *5* included 50-fold molar excess of unlabeled NRE oligonucleotide and unlabeled mutant NRE oligonucleotide, respectively. Incubation with 2 μg of anti-NRL antibody (*lane 6*) or 2 μg of normal rabbit IgG (*lane 7*) were used to detect the presence of NRL in the shifted protein complex. Experiments were repeated three times, and similar results were obtained. *C*, activation of the retinal *Mef2c* promoter in transfected HEK293 cells by NRL alone, CRX alone, and in combination. HEK293 cells were co-transfected with the *Mef2c* promoter driving luciferase-reporter construct (pGL3-*Mef2c*P-Luc), *Renilla* luciferase construct (pRL-CMV) for normalization, and expression plasmids for human NRL and/or human CRX. Cells were harvested 48 h after transfection for luciferase assays. Fold-activation was calculated relative to empty expression vectors. All experiments were repeated at least three times. Statistical comparisons by analysis of variance with Tukey-HSD post-analysis ($p < 0.01$ for all paired comparisons) are shown. *Bars* indicate S.D.

expression preceded *Rhodopsin* (*Rho*) and *Mef2c* transcripts (Fig. 3*C*).

Retinal Cell Type-specific Expression of Mef2c—To examine whether *Mef2c* is expressed specifically in photoreceptors, we examined its expression in *rd1* mice. This mouse strain is homozygous for a mutation in *Pde6b* and they lose almost all rod photoreceptors by age P33, but retain most of their cones and other non-photoreceptor neurons (58). By age P99, *rd1* retinas also lose cones and are photoreceptor-less. Gene expression assays for *Mef2c* transcripts (exons 5–6), *Nrl* (rod-specific marker), and M-opsin (*Opn1mw*, cone-specific marker) were performed for WT (P33), rod-less (*rd1,* P33), and photoreceptor-less retinas (*rd1*, P117) (Fig. 3*D*). Similar to *Nrl*, *Mef2c* transcripts were detected in mature WT retina but not in rod-less or photoreceptor-less retinas. Additionally, we could not detect *Mef2c* transcripts (exons 5–6) or *Nrl* transcripts in the $Nrl^{-/-}$ retina at age P28, whereas both were abundant in WT retina at P28 (data not shown). The *rd1* and $Nrl^{-/-}$ data suggest that *Mef2c* transcripts starting from exon 4 are specific to rod, and not cone, photoreceptors.

In Vivo and in Vitro Association of NRL with the Retinal Mef2c Promoter—A number of observations implicate NRL as an excellent candidate for controlling the activity of an alternative retinal *Mef2c* promoter; these include the timing of *Mef2c* expression specifically in rod photoreceptors and *in vivo* association of NRL immediately upstream of exon 4. ChIP-seq analysis detected NRL association overlapping with the location of Pol II binding, upstream of exon 4, in the adult mouse retina (see Fig. 1*B*). Further examination of this promoter region by ChIP assays detected NRL binding at P28 but not at P2 (Fig. 4*A*). NRL did not bind to a negative control region (*Rho*-neg) in wild type retina. NRL binding was not detected in $Nrl^{-/-}$ retina at either P28 or P2, confirming the specificity of the ChIP assay (Fig. 4*A*).

In silico analysis of this retinal *Mef2c* promoter region revealed the presence of a putative NRE: TGGCTCAG. To test whether NRL can directly bind to the region of interest, EMSA were carried out using an oligonucleotide probe (*Mef2c* oligo) spanning the NRE in the retinal *Mef2c* promoter (Fig. 4*B*). Nuclear extracts from HEK293T cells transfected with *Nrl* cDNA (NRL^+ NE) shifted the ^{32}P-labeled *Mef2c* oligo, whereas nuclear extracts from cells lacking NRL (NRL^- NE) did not, suggesting that NRL could bind to the *Mef2c* oligo (Fig. 4*B*, *lanes 1–3*). Specificity of the binding was validated by competition with an excess of unlabeled *Mef2c* oligo. An excess of the NRE mutant oligo did not compete for the binding (Fig. 4*B*, *lanes 4* and *5*). Consistent with these results, an anti-NRL antibody abolished the gel shift, whereas normal IgG did not (Fig. 4*B*, *lanes 6* and 7), further confirming the direct binding of NRL to the retinal *Mef2c* promoter.

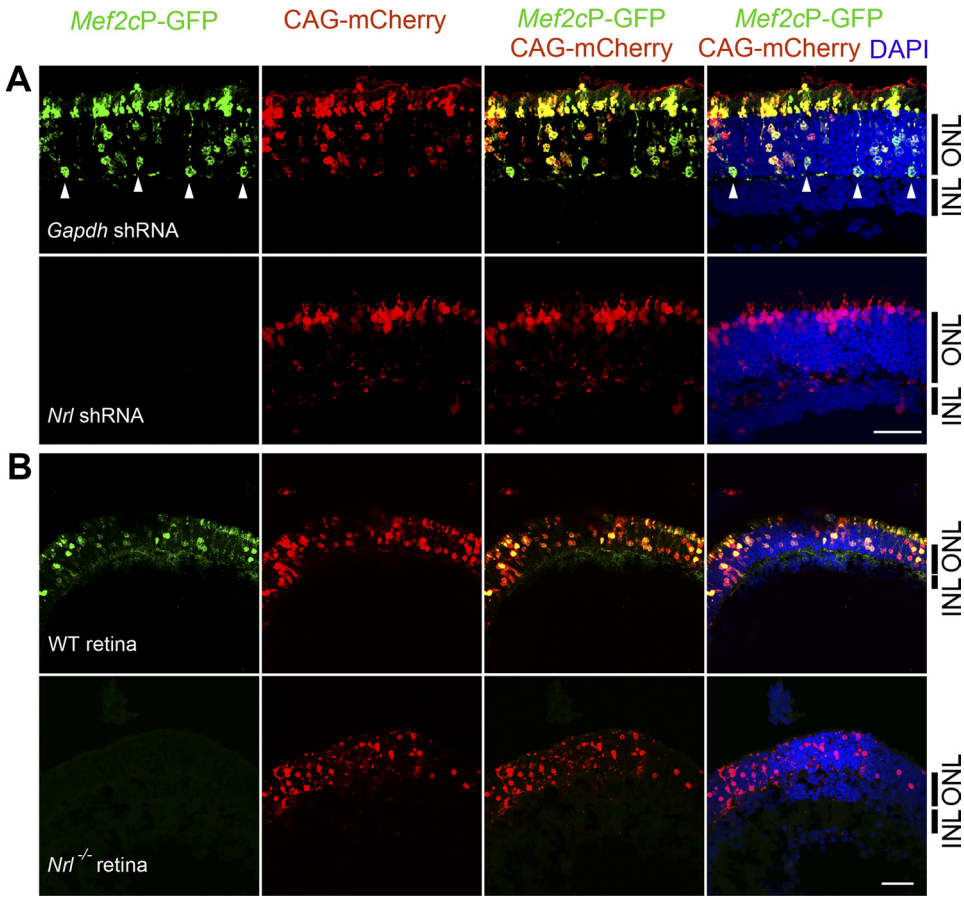

FIGURE 5. **Induction of retinal *Mef2C* promoter activity by NRL in mature rod photoreceptors.** *A*, representative confocal images of P28 CD1 wild type mouse retinas that were electroporated *in vivo* at P0 with *Mef2c*P-GFP, CAG-mCherry (electroporation control), and either *Gapdh* shRNA (top row) or *Nrl* shRNA (bottom row). Photoreceptor cells reside in the ONL but not in the inner nuclear layer (*INL*). *B*, representative confocal images of P20 C57BL/6J mouse retina (*top row*) and P20 $Nrl^{-/-}$ mouse (on C57BL/6J background) retina (*bottom row*) that were electroporated *in vivo* at P0 with *Mef2c*P-GFP and CAG-mCherry (electroporation control). *Mef2c*P-GFP expression is shown in *green* and CAG-mCherry in *red*. DAPI staining is in *blue*. Activation of the *Mef2c*P-GFP reporter in rod-photoreceptor cell bodies is indicated (*white arrowheads*). At least 3 biological replicates were collected for each electroporation condition and similar phenotypes were observed. *Scale bar*, 20 μm.

NRL Activates the Retinal Mef2c Promoter in Vitro and in Vivo—As a functional test of the ability of NRL to activate transcription from the retinal *Mef2c* promoter, we cloned a 540-bp region including a part of exon 4 into the pGL3 Firefly luciferase reporter vector. This construct (pGL3-*Mef2c*P-Luc) was co-transfected with or without an NRL expression plasmid into HEK293 cells. The *Mef2c* promoter was able to drive luciferase expression, and its activity was significantly enhanced by NRL ($p < 0.01$, analysis of variance) in a dose-dependent manner (Fig. 4*C*). NRL and CRX often cooperate to induce gene expression in mature rod photoreceptors (17, 59, 60), so we also tested the ability of CRX to activate the retinal *Mef2c* promoter (14, 58). CRX could activate the promoter alone and produced additional activation with NRL (Fig. 4*C*).

To examine whether NRL is able to activate the *Mef2c* promoter *in vivo*, we subcloned the same retinal *Mef2c* promoter into a GFP vector. This *Mef2c*P-GFP construct was transfected into mouse retinas at P0 by *in vivo* electroporation, and the promoter activity was monitored at P20 and P28 (Fig. 5). We observed similar results at P20 and P28. To confirm successful electroporation and normalize for transfection efficiency, a plasmid expressing mCherry driven by the CAG promoter (CAG-mCherry) was electroporated with *Mef2c*P-GFP. *Nrl* shRNA was co-electroporated into CD1 WT mouse pups to knockdown *Nrl* expression (46). Confocal imaging of retinal sections revealed that only photoreceptor cells, comprising the outer nuclear layer (ONL), expressed GFP (Fig. 5*A*). Expression of GFP was abrogated by co-electroporation with *Nrl* shRNA, but not with *Gapdh* shRNA, demonstrating that NRL is a key activator of the *Mef2c* promoter *in vivo* (Fig. 5*A*). To further evaluate whether the *Mef2c* promoter is expressed only in rod photoreceptors and is dependent on NRL, *in vivo* electroporation was carried out in cone-only $Nrl^{-/-}$ mice (Fig. 5*B*). We observed GFP expression only in sections of P20 WT retina, but not in P20 $Nrl^{-/-}$ retina (Fig. 5*B*).

Mef2c Activates the Rhodopsin Promoter in Vivo—To examine whether *Mef2c* can regulate rod photoreceptor-specific genes, we knocked down endogenous *Mef2c* expression in the retina by *in vivo* shRNA electroporation. To test the efficacy of *Mef2c* shRNA constructs, we first cotransfected HEK293 cells with a *Mef2c* expression construct (*Mef2c* cDNA) and *Gapdh* shRNA (control) or *Mef2c* shRNA. Knockdown efficiency was evaluated by immunoblotting (Fig. 6*A*), and the more efficient *Mef2c* shRNA construct (shRNA-2) was used for *in vivo* co-electroporation of the retina with a *Rhodopsin*-promoter fluorescence reporter construct (Rho-TdT). Electroporation of

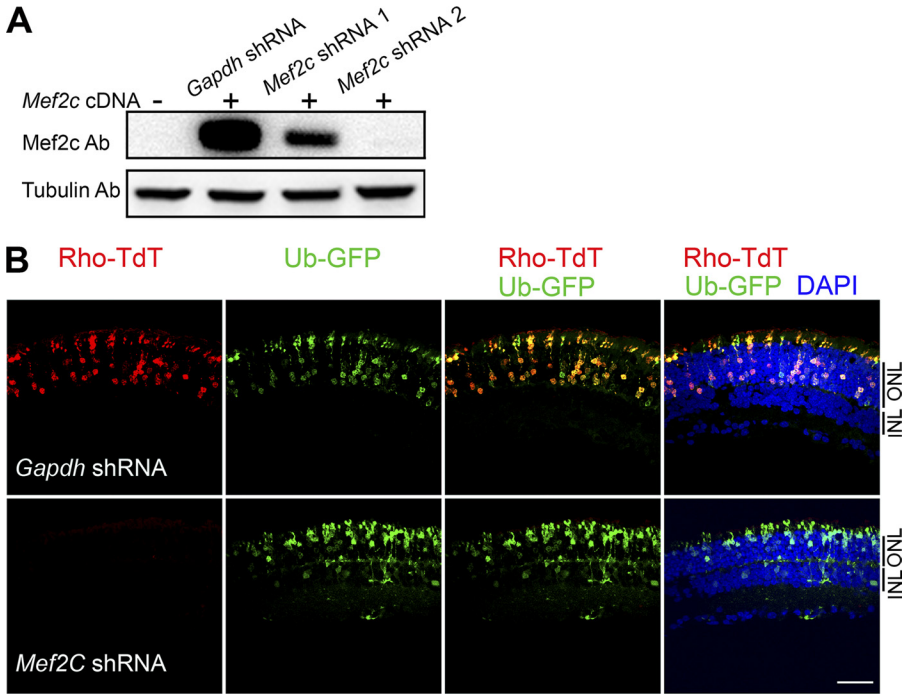

FIGURE 6. **Reduced *Rhodopsin* promoter activity by *Mef2c* knockdown in adult retina.** *A,* efficacy of *Mef2c* shRNA was validated in transfected HEK293 cells. HEK293 cells were co-transfected with the mouse *Mef2c* expression plasmid (Mef2c cDNA), and *Gapdh* shRNA or *Mef2c* shRNA 1 or *Mef2c* shRNA 2. Cells were harvested 48 h after transfection and lysed for immunoblot analysis with Mef2c antibody. Anti-tubulin antibody was used as loading control. *B*, knockdown of *Mef2c* by shRNA decreased *Rhodopsin* promoter activity in adult mouse retina. Representative confocal images of P20 CD1 wild type mouse retinas that were electroporated *in vivo* at P0 with the *Rhodopsin* promoter-Td tomato construct (Rho-TdT), ubiquitin promoter-GFP construct (Ub-GFP) (electroporation control), and either *Gapdh* shRNA (*top*) or *Mef2c* shRNA (*bottom*). Ub-GFP is shown in *green*. Rho-TdT is shown in *red*. DAPI is shown in *blue*. Photoreceptor cells reside in the ONL. At least 3 biological replicates were collected for each electroporation condition and a similar phenotype was observed. *Scale bar,* 20 μm.

Rho-TdT resulted in photoreceptor-specific expression of TdT that was restricted to the ONL (Fig. 6*B*). *In vivo* co-electroporation of *Mef2c* shRNA reduced the TdT expression driven from the *Rhodopsin* promoter, whereas *Gapdh* shRNA (control) had no effect on the TdT expression (Fig. 6*B*).

DISCUSSION

Spatiotemporal control of gene expression dictates development and homeostasis (1). Transcriptional promoters and enhancers are key determinants in the transcription of any gene by serving to integrate *cis*-regulatory DNA elements, transcription factor binding, epigenetic regulation, and signal transduction events (4). Alternative promoter usage is an important contributor to tissue and cell-specific gene expression (61, 62). In this study, we demonstrate that *Mef2c* expression in photoreceptors is regulated by NRL through a novel alternative promoter upstream of a 4th exon. The major lines of evidence are: 1) correlation of the expression profile of *Mef2c* with Pol II binding and increased H3K9 acetylation at the alternate *Mef2c* promoter in WT retina, 2) decreased *Mef2c* expression in $Nrl^{-/-}$ retina, 3) *in vivo* association of NRL with the *Mef2c* promoter and direct *in vitro* binding of NRL with the NRE within this promoter, and 4) NRL-mediated activation of the *Mef2c* alternate promoter in HEK293 cells and in the neural retina *in vivo*.

Our *in vivo* electroporation data show that the activation of the retinal-specific *Mef2c* promoter was restricted to the ONL of the mature retina, and included rod photoreceptors. The ONL is comprised of rod and cone photoreceptor cell bodies, with the cones restricted to the outermost (scleral) side (63, 64). Failure to detect GFP expression in the cone-only $Nrl^{-/-}$ retina strongly suggests that the retina-specific alternative *Mef2c* promoter is activated by NRL and only in rod photoreceptors.

Expression of *Mef2c* from the alternative promoter in the retina after age P16 correlated with the association of NRL and Pol II, as indicated by ChIP-qPCR in the mature retina (P28 or P25) but not in the developing retina (P2). A similar *Mef2c* promoter construct was reportedly inactive at P8 in electroporated mouse retinal explants (29). These observations support the hypothesis that the retina-specific *Mef2c* promoter is activated relatively late, in mature rod photoreceptors. We have previously shown that a minimal transactivation domain of NRL interacts with TATA-binding protein (65). It is possible that NRL activates *Mef2c* transcription by recruiting or stabilizing the TATA-binding protein and consequently the general transcription machinery at the *Mef2c* promoter. The mechanism underlying "late" induction of the retinal *Mef2c* promoter, despite the presence of NRL and CRX in rod progenitor cells, remain to be elucidated.

CRX activates the transcription of photoreceptor-specific genes (including all Opsins) by recruiting co-activators possessing histone acetyltransferase activity (65). In $Crx^{-/-}$ mice, the association of histone acetyltransferase-containing co-activators and acetylated histone H3 at *Opsin* promoters are decreased significantly, which correlates with diminished *Opsin* expression (65). Increased acetylation of histone H3, on Lys-9 or Lys-14, has been associated with enhanced transcrip-

tion, whereas histone hypoacetylation is correlated with transcription silencing (66). CRX association at the retina-specific *Mef2c* promoter region was recently reported in the mature mouse retina (29). Our H3K9-Ac ChIP-qPCR analysis demonstrates that acetylation of H3K9 increases substantially at the *Mef2c* alternative promoter, suggesting the transition of this chromatin region from a closed architecture to an open architecture within mature photoreceptors. Thus, it is possible that CRX recruits histone acetyltransferase-containing co-activators to the retinal-specific *Mef2c* promoter, promoting an open chromatin architecture that facilitates NRL binding and recruitment of the basal transcription machinery. This is consistent with the observation that CRX alone was not sufficient to activate the *Mef2c* promoter as shown by our *in vivo* electroporation results of $Nrl^{-/-}$ mice or shRNA knockdown of *Nrl* expression in WT mice.

Elucidation of the NRL-centered gene regulatory network, which dictates photoreceptor cell fate and function, requires identification of the transcriptional targets of NRL. Most importantly, transcription factor targets represent critical secondary nodes that regulate the expression of distinct or shared downstream target genes. Gene expression profiles from $Nrl^{-/-}$ mice continue to accelerate this discovery process (33, 35, 67).

The transcription factor *Mef2c*, reported here as a novel NRL target in the rod photoreceptor, was previously shown to regulate cell specification and survival in multiple tissues. *Mef2c* knockdown by shRNA, using *in vivo* electroporation, significantly decreased *Rhodopsin* promoter activity within rod photoreceptors. Hence, *Mef2c* might play an important role in regulating rod homeostasis by participating in the regulation of specific genes. Further investigations are required to identify the downstream targets of MEF2C in rod photoreceptors. Our studies, however, implicate MEF2C as an important regulator of rod gene expression downstream of NRL.

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