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Nr2e3-Directed Transcriptional Regulation of Genes Involved in Photoreceptor Development and Cell-Type Specific Phototransduction

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Abstract

The retinal transcription factor *Nr2e3* plays a key role in photoreceptor development and function. In this study we examine gene expression in the retina of *Nr2e3^{rd7/rd7}* mutants with respect to wild-type control mice, to identify genes that are misregulated and hence potentially function in the *Nr2e3* transcriptional network. Quantitative candidate gene real time PCR and subtractive hybridization approaches were used to identify transcripts that were misregulated in *Nr2e3^{rd7/rd7}* mice. Chromatin immunoprecipitation assays were then used to determine which of the misregulated transcripts were direct targets of NR2E3. We identified 24 potential targets of NR2E3. In the developing retina, NR2E3 targets transcription factors such as *Ror1*, *Rorg*, and the nuclear hormone receptors *Nr1d1* and *Nr2c1*. In the mature retina NR2E3 targets several genes including the rod specific gene *Gnb1* and cone specific genes blue opsin, and two of the cone transducin subunits, *Gnat2* and *Gnb3*. In addition, we identified 5 novel transcripts that are targeted by NR2E3. While mislocalization of proteins between rods and cones was not observed, we did observe diminished concentration of GNB1 protein in adult *Nr2e3^{rd7/rd7}* retinas. These studies identified novel transcriptional pathways that are potentially targeted by *Nr2e3* in the retina and specifically demonstrate a novel role for NR2E3 in regulating genes involved in phototransduction.

Introduction

Enhanced-S-Cone Syndrome (ESCS) is an autosomal recessive retinopathy caused by mutations in the orphan receptor NR2E3 (Haider et al., 2000; Nakamura et al., 2004). While most hereditary human retinal diseases affect the topography of mature photoreceptor cells through apoptotic loss of cells, ESCS manifests as a gain of function in S (short, blue) cone cells; the least populous of the photoreceptor cells (Haider et al., 2000). Patients also exhibit early night blindness, retinal tearing, neovascularization, and varying degrees of L (long, red)

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cone and M (middle, green) cone vision and of retinal degeneration (Jacobson et al., 1990; Marmor et al., 1990; Jacobson et al., 1991; Haider et al., 2000; Milam et al., 2002; Jacobson et al., 2004). Mutations within NR2E3 are also associated with a related retinal disease, Goldman Favre syndrome, clumped pigmentary retinopathy, and more recently in autosomal dominant retinitis pigmentosa as well as a milder form of ESCS (Favre, 1958; Fishman et al., 1976; Gerber et al., 2000; Sharon et al., 2003; Chavala et al., 2005; Hayashi et al., 2005; Coppieters et al., 2007; Lam et al., 2007; Gire et al., 2007).

The retinal degeneration 7 (*rd7*, *Nr2e3^{rd7/rd7}*) mutant mouse which lacks NR2E3, has been shown to have an increase in blue opsin expression without a concomitant increase in green opsin or significant reduction in rhodopsin expression (Hawes et al., 1999; Akhmedov et al., 2000; Haider et al., 2001). Our recent studies show that the increase in blue opsin expressing cones of the *Nr2e3^{rd7/rd7}* mouse results from abnormal proliferation due to a defect in mitotic progenitors to suppress their cone generation program (Haider et al., 2006). Persistent ectopic progenitors are thus likely to lead to the gain of S-cone function observed in ESCS patients.

While earlier whole genome studies have identified genes that are differentially expressed in *Nr2e3^{rd7/rd7}* retinas compared to controls (Chen et al., 2005; Corbo and Cepko, 2005; Peng et al., 2005; Cheng et al., 2006) the transcriptional network through which NR2E3 functions still remains poorly understood. While *Nr2e3* action alone does not have a profound effect on rod cell differentiation, in combination with *Nrl*, it promotes rod development (Hennig et al., 2008; McIlvain and Knox, 2007). Recent studies suggest that *Nr2e3* functions as a dual transcriptional regulator and lack of *Nr2e3* leads to hybrid photoreceptor cells that de-repress cone genes and reduce rod gene expression (Corbo and Cepko 2005; Cheng et al., 2006, Webber et al. 2008). In this study we identified differentially expressed genes by subtractive hybridization and performed quantitative real time PCR (qRT-PCR) to evaluate those as well as over 100 genes that have a putative or known function in the development or maintenance of the retina, or for which mutations are associated with human retinal disease. We further used chromatin immunoprecipitation to identify which of the differentially expressed genes *Nr2e3* directly regulated. This study was performed at two postnatal time points: P2 and P21. P2 was chosen as a developmental time point during which *Nr2e3* is expressed. We chose P21 to examine genes differentially expressed and targeted by *Nr2e3* in the adult retina. Differential gene expression in the adult may occur as a secondary effect of retinal dysplasia apparent in the *rd7* mouse at P21, however differentially expressed genes that are direct targets of NR2E3 likely contribute the adult *rd7* phenotype. Through our comprehensive study, we identified genes potentially regulated by *Nr2e3* such as the nuclear receptor *Nr1d1*, which was previously reported to be a co-factor of *Nr2e3* (Cheng et al., 2004), the cone specific phototransduction genes blue opsin and the α and β cone transducin subunits *Gnat2* and *Gnb3* respectively, and the rod specific gene, guanine nucleotide binding protein 1 (*Gnb1*). While the expression level of these genes is altered in *Nr2e3^{rd7/rd7}* mice, at the message and protein level, they are not mislocalized to other cells. This suggests that NR2E3 functions by regulating genes in a temporal and cell-type specific manner in independent transcriptional networks in rod and cone cells.

Materials and Methods

Animals

The mice used in this study were bred and maintained under standard conditions in the research vivariums at The Jackson Laboratory and at the University of Nebraska Medical Center. Tissues were harvested from B6.Cg-*Nr2e3^{rd7/rd7}* and C57BL/6J (B6) at the following postnatal (P) 2, and P21. A minimum of three *Nr2e3^{rd7/rd7}* and three B6 mice were analyzed for each time point. Mice were genotyped for the *Nr2e3^{rd7/rd7}* mutation as previously described (Haider

et al., 2001). Adult mice were phenotyped by indirect ophthalmoscopy for the characteristic *Nr2e3^{rd7/rd7}* panretinal spots prior to additional analysis.

Subtractive Hybridization

Subtractive hybridization was performed using Clontech PCR-Select cDNA Subtraction kit (BD biosciences Clontech) per manufacturer's instructions. Briefly, total RNA was isolated from P2 and P21 eyes of control and *Nr2e3^{rd7/rd7}* mice. Three pooled samples (n=10 eyes in each pool) were generated for each genotype and time point. cDNAs were synthesized from each sample and tester and driver cDNAs were digested with *Rsa*I to generate blunt ended molecules. The tester cDNAs were then subdivided into two portions and each was ligated with a different cDNA adaptor. Two hybridizations were performed for each tester cDNA to normalize differentially expressed genes and then to generate PCR templates from differentially expressed sequences. PCR amplified products were cloned into Topo-XL and sequenced. All clones (approximately 200) were sequenced and analyzed using Sequencher (V. 4.6, GeneCodes, Ann Arbor, MI) and NCBI nucleotide BLAST.

Real time PCR

Total RNA was isolated from seven P2 and P21 pairs of eyes from *Nr2e3^{rd7/rd7}* and control (C57BL/6J, B6) mice using Trizol. Two µg of total RNA was reverse transcribed in independent triplicates using Retroscript (Ambion). cDNA samples were diluted 1:100 and real time PCR was performed in triplicate for each primer using SYBR Green PCR Master Mix (Applied Biosystems). PCR products of approximately 200 nucleotides were amplified with primers selected using Primer Express 2.0 software program. Reactions were quantified using an ABI 7500 Real Time PCR instrument and analyzed with accompanying software. Relative expression levels were determined by normalizing cycle threshold values for each primer to the amount of β -Actin expressed ($1000/2^{(Ct\text{-gene}-Ct\text{-}\beta\text{-Actin})}$). Relative fold change was calculated from normalized values. Significant expression differences were assessed by the student's T-Test with a minimum p-value of 0.05. Supplemental Table 1 (S1) lists all primers used for real time PCR.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed using reagents from the ChIP kit (Upstate, Millipore) and a modified protocol. Briefly, retinas were dissected from P2 or P21 C57BL/6J eyes (8–10 eyes per ChIP; three independent assays per time point) and placed into 400 µl of PI buffer (1 tablet protease inhibitor cocktail, Roche) in 10ml phosphate buffered saline (PBS) on ice. Chilled retinas were dissociated and crosslinked in 37% formaldehyde (1% final concentration) for 20 minutes at room temperature on a rotating platform. Retinas were incubated for an additional 5 minutes in 1M glycine (final concentration 0.125mM) and rinsed twice with PI buffer before lysis. Retinas were lysed on ice and sonicated on ice using a Misonix Sonicator 3000 (power 5, pulse 10×, 30 second pause between pulses; 150 total pulses). Retina lysates were pre-cleared using a salmon sperm DNA/protein A Agarose-50% slurry and immunoprecipitation was performed overnight with approximately one µg of NR2E3 antibody (Haider et al. 2006) at 4°C with rotation. Immunoprecipitated samples were eluted with 1% SDS, 0.1M NaHCO₃ buffer and reverse cross-linked with NaCl (200 mM final concentration) and incubated with 10 mg RNase A at 65°C for five hours. Input (control) samples were isolated similarly and incubated with pre-clear solution instead of antibody or with goat IgG antibody (negative control). PCR reactions were performed using 1 µl of 1:10 diluted Input or Experimental (NR2E3 precipitated) sample under standard PCR conditions as previously described (Haider et al., 2001) using an annealing temperature of 58°C and 35 cycles. PCR reactions were electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining. Primers approximately 100 nucleotides 5' or 3' from a putative response

element sequence were selected to amplify approximately 200 base pair products (bp) (Supplemental Table 2, S2).

Immunohistochemistry

Immunohistochemical analysis was performed on 6 μ m serial sections of B6 and *Nr2e3^{rd7/rd7}* eyes as previously described (Haider et al. 2006). Briefly, paraffin embedded eyes were fixed with either 4% PFA or Methanol/Acetic acid (3:1) and maintained at a dorsal/ventral orientation. After blocking with 2% normal goat or horse serum (Vector, CA) in PBS, sections were incubated with the following primary antibodies: Gnb1 (rabbit, 1:100, Protein Tech Group), rhodopsin (mouse, 1:500, Leinco Technologies), peanut agglutinin (1:200, Sigma) at 4°C overnight. The next day, samples were rinsed and incubated in the appropriate secondary antibody (1:400, AlexaFluor by Molecular Probes, Invitrogen) for 1–2 hours. Images from sections were collected on a Zeiss Axioplan II fluorescent microscope equipped with the appropriate bandpass filters for each fluoroChrome.

Results

Gene expression analysis in the developing and mature retina of *Nr2e3^{rd7/rd7}* mice

To characterize the transcriptional networks associated with NR2E3 function in developing and mature photoreceptors, we determined the expression profile of genes in mice lacking *Nr2e3*. Subtractive hybridization was performed using P2 and P21 retina samples to identify genes involved in development and maintenance of photoreceptors. At each time point, B6 cDNAs were subtracted from *Nr2e3^{rd7/rd7}* cDNAs to identify transcripts up-regulated in *Nr2e3^{rd7/rd7}* mice, and conversely, *Nr2e3^{rd7/rd7}* cDNAs were subtracted from B6 cDNAs to identify the transcripts which are down-regulated in *Nr2e3^{rd7/rd7}* mice. We sequenced approximately 200 clones and identified 14 up-regulated and 12 down-regulated transcripts at P2, and 10 up-regulated and 6 down-regulated transcripts at P21 (Supplemental Table S3). The observed expression changes identified through subtractive hybridization were then confirmed by real time PCR.

Additionally, we took a more targeted approach and performed real time PCR testing over 100 genes known to function in photoreceptor development, structure or maintenance of the retina, in transport of proteins in the retina, and approximately 20 orphan receptors, to determine if any known retinal genes were misregulated in *Nr2e3^{rd7/rd7}* mice (Supplemental Table S4). Overall, from this combined effort, 30 genes at P2 and P21 were found to be misregulated in the *Nr2e3^{rd7/rd7}* compared to wildtype retinas (Figure 1, Table 1). While 10 of these genes were also reported in previous studies (Corbo and Cepko 2005, Chen et al, 2005 Oh et al. 2008) only three were previously identified as targets of NR2E3 (Peng et al. 2005). At P2, thirteen genes were significantly up regulated and three genes that are downregulated in *Nr2e3^{rd7/rd7}* retinas relative to controls. The majority of these genes were transcriptional regulators, four were signaling molecules, and several novel transcripts with unknown function were also identified. Six of the sixteen genes were identified by both subtractive hybridization and targeted expression profiling approaches. At P21, we identified twenty misregulated genes. In contrast to the genes identified at P2, the majority of these genes were not transcription factors. Expression of several signaling molecules were altered in mutant retinas. Also, while most of the genes identified at P2 were up-regulated, an equal number of genes were either up or down regulated at P21 in the *Nr2e3^{rd7/rd7}* retinas relative to controls. Six genes were differentially expressed at P2 and P21.

Potential targets of *Nr2e3* include transcription factors and phototransduction genes

In order to determine which of the misregulated genes were potential targets of *Nr2e3*, we performed chromatin immunoprecipitation (ChIP) using P2 and P21 B6 retinas. First, we

evaluated approximately 30 kb of 5' untranslated sequence from each of the 30 misregulated genes (from Figure 1, Table 2) to identify potential *Nr2e3* binding/response element sequences. These sites were chosen based on earlier reports (Kobayashi et al. 1999, Chen et al. 2005) that defined a putative response element for NR2E3 (AAGTCAnAAGTCA), which included two identical motifs separated by a spacer. At P2, ten putative targets NR2E3 binding sites were evaluated, and eight genes were identified as directly targeted by *Nr2e3* (Figure 2, Table 1). Among these genes were novel transcripts with unknown function (Riken cDNA gene 2610101N10 (Rik1n10), and accession id BC07393 (BC7393)), two were known transcription factors (*Ror1*, *Rorg*), and one was a signaling protein (*Irbp*). At P21, ten of the eleven binding sites we tested were positive (Figure 2). When examining the expression at P2 and P21, six genes were misregulated at both P2 and P21 (*Nr2e3*, *Nr1d1*, *Nr2c1*, *Rgr*, *Gnb3*, and *Rora*). Interestingly, NR2E3 appears to regulate its own expression in both the developing and mature retina (also in Peng et al. 2005). While many of the response elements we identified were not identical dimeric motifs, they were greater 75% similar to the putative target sequence. The spacer sequences varied from one to four nucleotides, with one RE sequence, for the gene *Ankmy2*, containing a perfect dimeric repeat with a 13-nucleotide spacer sequence.

Altered expression of transcripts translates to misexpression of key retinal proteins

To determine if misregulation of gene expression resulted in an alteration of temporal or spatial protein expression of proteins directly targeted by NR2E3, we performed immunohistochemical analyses for the rod specific gene GNB1. As expected, in control B6 retinas, GNB1 co-localized with rhodopsin (Figure 3A–C) and not with the cone cell marker peanut agglutinin (PNA) (Figure 3G–I). Further, in the *Nr2e3^{rd7/rd7}* retinas, GNB1 expression (Figure 3D–F, J–L) was restricted to rod outer segments, whereas in the B6 retina, expression was observed in both the outer and inner segments (Figure 3A–C, G–I). We did not observe mislocalization of GNB1 to cone cells in *Nr2e3^{rd7/rd7}* retinas. While altered protein localization and expression can be a secondary effect of the morphological changes apparent in the mature *rd7* retina, that *Gnb1* expression is potentially regulated by NR2E3 suggests that suggests in this case, altered expression may be due to *Nr2e3* function in the mature rod cell.

Discussion

Enhanced S-Cone Syndrome has the unusual phenotype of a gain of function of the least populous photoreceptor subtype. Although this is a rare human disorder, the nuclear receptor NR2E3, which is mutated in this disorder, is critical to photoreceptor development, and specifically photoreceptor generation. Our previous studies using *Nr2e3^{rd7/rd7}* mice as a model for ESCS show that the mechanism through which this phenotype occurs is over-proliferation of ectopic retinal progenitors (Haider et al., 2006). A similar enhanced S-cone phenotype is also observed in the *tlx* mice, lacking *Nr2e1*, a nuclear receptor very similar to *Nr2e3*. *Nr2e1* modulates retinal cell proliferation and cell-cycle re-entry by inhibiting *Pten* expression, a negative regulator of neural stem cell proliferation (Zhang et al., 2006; Sun et al., 2007).

In this study, we identified several genes whose expression is regulated by *Nr2e3* in the developing and mature retina. While the genes directly regulated by NR2E3 overlap to some extent in the developing and adult retina, the majority were unique subsets, suggesting a unique role for NR2E3 both in the development and maintenance of photoreceptor cells. Twenty-four direct targets of NR2E3 were identified, only three of which were previously reported as NR2E3 targets (Peng et al. 2005). Several classes of genes emerged suggesting NR2E3 provides input into multiple transcription networks simultaneously. These include transcription factors (e.g. *Nr1d1*, *Nr2c1*, and *Rorg*) and phototransduction genes (e.g. blue opsin, *Gnat2*, *Gnb1*, and *Gnb3*). In addition, we identified several novel genes contributing to these networks through regulation by NR2E3. While the majority of the genes we identified were up-regulated

in *Nr2e3^{rd7/rd7}* retinas, we did identify several downregulated targets, suggesting that *Nr2e3* can function both as a suppressor and/or enhancer of transcription. We identified several transcription factors targeted by *Nr2e3* in the developing retina. Further work will need to be done to determine whether these genes are specific to mitotic retinal progenitors or postmitotic, differentiating cells that are committed to a cone or rod lineage. In addition, at P2 *Nr2e3* regulates photoreceptor specific genes such as *Irbp*, *recoverin*, and *rhodopsin kinase*. While the eye is not yet open at P2, and the outer segments of photoreceptors, where these genes ultimately function, are still being developed, *Nr2e3* regulated gene expression apparently prepares the retina for visual transduction.

Recent studies have shown *Nr2e3* action alone does not have a profound effect on rod cell differentiation, however in combination with *Nrl* it promotes rod development (Hennig et al., 2008; McIlvain and Knox, 2007). Earlier gene expression studies have shown that lack of *Nr2e3* results in misexpression of rod and cone genes; and specifically downregulation of rod genes and upregulation of cone genes (Corbo and Cepko 2005; Cheng et al., 2006). Similar to these reports, our study confirms that in *Nr2e3^{rd7/rd7}* mice, cone specific genes such as blue opsin, and the alpha and beta transducins (*Gnat2* and *Gnb3*) are up-regulated while rod specific genes such as *Gnb1* are down regulated compared to normal. The altered mRNA expression level translates into altered protein expression for GNB1 as seen in this study as well as GNAT2 (Haider et al. 2006) and PRPH2/RDS (Nystuen et al. 2008). Additionally, we also observed several genes expressed in both rod and cone cells were differentially expressed in the adult *Nr2e3^{rd7/rd7}* retina. Interestingly, at P2, most of the differentially expressed genes were up-regulated in *Nr2e3^{rd7/rd7}* retinas compared to normal, while in the adult retina, an approximately equal number of genes were increased or decreased. The six potential NR2E3 targets at P2 and P21 are all up-regulated in the P2 retina however only 4 are up-regulated in the P21 retina (*Nr2e3*, *Nr2c1*, *Nr1d1*, *Gnb3*) while two are down-regulated (*Rgr*, *Rora*). The down-regulation of *Rgr* and *Rora* in the *Nr2e3^{rd7/rd7}* retina could be due to loss of photoreceptor cells and not a direct consequence of lack of *Nr2e3*. These results suggest that perhaps in the developing retina *Nr2e3* primarily functions as a repressor of transcription while in the adult retina it may function equally as a repressor or activator. Differential expression in the adult retina however may also be due to the primary developmental defects and thus do not accurately reflect how *Nr2e3* functions. Additional experiments to confirm which of the targets identified in this study are directly targeted by *Nr2e3* versus those that are indirect targets or those that are differentially expressed due to secondary factors will be essential. Further, we are examining *Nr2e3* function in a conditional mutant mouse where normal *Nr2e3* expression persists in the developing retina and loss of *Nr2e3* occurs only in mature rod or cone cells. These studies will provide valuable insight into delineating the role of *Nr2e3* in the mature retina. *In vitro* and gain of function studies using the *Nrl^{-/-}* mice suggest that *Nr2e3* functions with *Nrl* in rod development (Cheng et al., 2004; Cheng et al., 2006). Our current studies demonstrate *Nr2e3* potentially regulates the expression of rod and cone specific genes suggesting its role is not limited to suppression of cone genes in rod photoreceptor cells.

The molecular mechanisms through which *Nr2e3* functions to direct cone cell generation, rod differentiation, and visual transduction remain to be determined. As a member of the steroid hormone family of nuclear receptors, *Nr2e3* contains several conserved domains including DNA binding, dimerization, and ligand binding. The nuclear hormone receptor genes may function as heterodimers or homodimers and while many require ligand activation for function, they can also be recruited to the nucleus by cofactors. *Nr2e3* is in the class of orphan receptors for which no ligand has been identified to date. *Nr2e3* has been shown to interact with the nuclear receptor *Nr1d1* (Cheng et al., 2004) and more recently, a novel cell-cycle co-repressor was identified for *Nr2e3*, RetCoR that expresses in the brain and retina (Takezawa et al., 2007). *Nr2e3* appears to recruit RetCoR to repress *Cyclin D1* expression, which is required for proliferation of retinal progenitor cells. While there is currently no known endogenous ligand

for NR2E3, high throughput assays have been performed and identified agonists of NR2E3 (Kapitskaya et al. 2006, Wolkenberg et al. 2006). It is well established that two ligands, retinoic acid and thyroid hormone, play a significant role in the developing retina and in particular in the differentiation of photoreceptor cells, however they have not yet been associated with NR2E3. In fact, thyroid hormone levels appear normal in the *Nr2e3^{rd7/rd7}* retina (Yanagi et al. 2002) suggesting it is not the ligand for *Nr2e3*. Our previous studies show that NR2E3 expression in the developing retina appears to be entirely nuclear while there is nuclear and predominantly perinuclear staining in the mature retina (Haider et al. 2006). This would suggest that perhaps in the developing retina, this nuclear receptor may be constitutively active and not require ligand or co-factor for activation and localization to the nucleus while additional aid from ligand activator or co-factor recruitment may be required in the mature retina. ChIP data presented in this study showing NR2E3 recruitment to its own promoter site may suggest an autoregulatory mechanism is present and may contribute to its highest expression in the adult retina (our unpublished data) and its higher message levels in the *Nr2e3^{rd7/rd7}* retina. Additional studies to examine NR2E3 complexes in the developing versus mature retina will provide further understanding of how it is recruited to the nucleus. These findings support a multifunctional role for *Nr2e3* as dual repressor/activator to regulate retinal progenitors as well as postmitotic rod and cone cells.

This study provides new information about the transcriptional networks that regulate photoreceptor generation and function. The identification of transcription factors that are direct targets for *Nr2e3* provides entry points into the biological pathways regulating photoreceptor generation and possibly differentiation. These studies also suggest a novel function for *Nr2e3* in the mature retina in regulation of phototransduction genes in a cell dependent manner. Further studies will be performed to determine the cell type specific regulation of the *Nr2e3*-regulated genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Genes misregulated in the developing and mature *Nr2e3^{rd7/rd7}* retina
 Relative fold change of genes misregulated in *Nr2e3^{rd7/rd7}* retinas compared to control, B6, retinas. Panel A are genes misregulated at P2, Panel B are genes misregulated at P21. Standard errors are indicated for each gene. All genes met a minimum significance of $p \leq 0.05$.

Figure 2. Direct targets for NR2E3

Chromatin immunoprecipitation assays to identify direct targets of NR2E3 at P2 and P21. Genes for each amplicon are indicated at the bottom of each gel. Marker (100 base pair ladder, Roche), I (input DNA, positive control) +NR2E3 (NR2E3 precipitated chromatin sample), +IgG (negative control, goat IgG precipitated chromatin) Mock (negative, no chromatin control).

Figure 3. NR2E3 directly regulates rod specific genes that are not misexpressed in *Nr2e3^{rd7/rd7}* retinas

P30 B6 and *Nr2e3^{rd7/rd7}* retinas labeled for GNB1 in rod photoreceptor cells. A–C: B6 retina double labeled with (A) GNB1 and (B) rhodopsin, and (C) merged image shows co-localization of both GNB1 and rhodopsin with DAPI nuclear stain (blue). D–F: *Nr2e3^{rd7/rd7}* retina double labeled with GNB1 (D) and rhodopsin (E), and merged image with GNB1, rhodopsin, and DAPI nuclear stain (blue) (F) shows co-localization of both proteins. G–I: B6 retina double labeled with GNB1 (G) and cone marker PNA (H), and merged image with GNB1, PNA, and DAPI nuclear stain (blue) (I) shows no co-localization. Inset is a higher magnification view of cells indicated with asterisks. J–L: *Nr2e3^{rd7/rd7}* retina double labeled with GNB1 (J) and cone marker PNA (K), and merged image with GNB1, PNA, and DAPI nuclear stain (blue) (L) shows no co-localization. Inset is a higher magnification view of cells indicated with asterisks.

GNB1 is expressed in both the inner and outer segments of B6 retina; expression appears to be restricted to the outer segments in *Nr2e3^{rd7/rd7}* retinas.

Table 1

Genes differentially expressed in the *Nr2e3^{rd7/rd7}* developing and mature retina

Genes identified first by subtractive hybridization (SH) are indicated as +; chromatin immunoprecipitation (ChIP) data is indicated as + or – for at either P2 or P21 or P2 and P21; genes that were identified in other expression studies using *Nr2e3^{rd7/rd7}* mice are referenced as well as three genes that were previously identified as targets of NR2E3. NA (no RE sequence available).

Gene name	Gene symbol	Function	SH	ChIP	Previously identified as differentially expressed	Previously identified as direct target	Reference (s)
Receptor tyrosine kinase-like orphan receptor 1	Ror1	Cell signaling		+ P2	No	No	
RAR-related orphan receptor gamma	Rorg/c	Cell differentiation, transcription factor		+ P2	No	No	
retinal G protein coupled receptor	Rgr	Signaling, phototransduction		+P2/P21	No	No	
RAR-related orphan receptor alpha	Rora	Cell differentiation, transcription factor		+P2/P21	No	No	
Nuclear receptor subfamily 2, group C, member 1	Nr2c1	Transcription factor		+P2/P21	No	No	
Nuclear receptor subfamily 1, group D, member 1	Nr1d1	Transcription factor		+P2/P21	No	No	
Nuclear receptor subfamily 2, group E, member 3	Nr2e3	Transcription factor photoreceptor development		+P21	Yes	Yes	Peng et al 2005 Oh et al 2008
Guanine nucleotide binding protein (G protein), beta polypeptide 3	Gnb3	Signal transduction		+P2/P21	Yes	No	Chen et al. 2005 Corbo et al 2005
Interphotoreceptor retinoid binding protein	Irbp/Rbp3	Phototransduction	+	+ P2	No	Yes	Peng et al 2005
Riken 2610008E11	Rik8e11	Unknown	+	– P2	No	No	
Riken 261010N10	Rik1n10	Unknown	+	+P21	No	No	
Mitochondrial ribosomal protein L18	Mrp18	Translation	+	+ P2	No	No	
BC037393, NEDD4212	BC7393	Novel	+	+P2	No	No	
Recoverin	Rcvn	Phototransduction		+ P2	No	No	
Rhodopsin kinase	RhoK	Phototransduction		+ P2	No	No	
KH domain containing RNA binding signal protein	Khdrbs3	Signal transduction	+	– P2	No	No	
ATP-binding cassette, sub-family C member 6	Abcc6	Phototransduction		+P21	No	No	

Gene name	Gene symbol	Function	SH	ChIP	Previously identified as differentially expressed	Previously identified as direct target	Reference (s)
Guanylate cyclase activator 1A	Gucal1a	Phototransduction		+P21	Yes	No	Corbo et al 2005
guanine nucleotide binding protein, alpha transducing 2	Gnat2	Phototransduction		+P21	Yes	No	Chen et al. 2005 Corbo et al 2005 Oh et al 2008
opsin 1 (cone pigments), short-wave-sensitive	Bops	Phototransduction	+	+P21	Yes	Yes	Chen et al. 2005
opsin 1 (cone pigments), short-wave-sensitive	Bops	Phototransduction	+	+P21	Yes	Yes	Chen et al. 2005 Corbo et al 2005 Oh et al 2008 Peng et al 2005
tuberous sclerosis 1	Tsc1	Chaperone Protein		-P21	No	No	
ankyrin repeat and MYND domain containing 2	Ankmy2	Ion binding	+	+P21	No	No	
neural retina leucine zipper gene	Nrl	Transcription factor photoreceptor development		-P21 (weak)	Yes	No	Chen et al 2005 Oh et al 2008
ATP-binding cassette, subfamily A (ABC1), member 4	Abca4	Phototransduction		+P21	No	No	
guanine nucleotide binding protein (G protein), beta 1	Gnb1	Signal transduction	+	+P21	Yes	No	Oh et al 2008
crumbs homolog 1 (Drosophila)	Crb1	Photoreceptor development, membrane organization		+ P215	Yes	No	Corbo et al 2005
nuclear protein 1	Nuprl	Cell growth	+	+ P21	Yes	No	Chen et al 2005
crystallin, alpha A	Cryaa	Maintain structure	+	NA			
crystallin, gamma B	Crygb	Promote neurite formation	+	NA	Yes	No	Chen et al 2005

Table 2
Response element sequences within regions identified as potential NR2E3 targets by ChIP assay

Response elements were identified from approximately 30 kilobase (kb) regions of 5' untranslated sequence from genes that were misregulated in *Nr2e3^{rd7/rd7}* retinas. Sequences are listed only for the genes that were shown to be positive by chromatin immunoprecipitation. Sequence homology describes the percent homology of the region surrounding the response element as determined using VISTA genome browser. Asterisks denotes the 5 genes whose RE sequences lie within the gene.

Gene Symbol	>50% homology to human sequence	RE sequence	Distance from ATG (base pair)
P2			
Rorg		CCCTCAAAGTCA	-1318
Irbp/Rbp3	✓	TGACTTGTCAT	-5877
Ror1	✓	AAGTCACAACTGA	-22987
Mrpl18	✓	AAAACGGAAGTCA	-255
Rik1n10		TGACTTTGCTGGCTT	-5717
Bc7393		TGACTTCAATTACAT	-10942
Rcvn		TGTTTGTACTT	-1401
RhoK		TGCCTTCTGAGTG	-4889
P21			
Abcc6 (*)		AGTCTTTTGTACTC	-3205
Guca1a (*)		AAATCAAAAGTC	-33347
Gnat2	✓	AAGTCACTAAGCAC	-2390
Opsin1sw		TGACTTTTCCATT	-9532
Nupr1 (*)	✓	AATGCAGGAGATAAAGTCA	1236
Nrl		TTACTTTTGAATC	-15857
Abca4	✓	AAGTCAAAAGAA	-9286
Gnb1		GAGTGAAAAAGTCA	-36579
Crb1	✓	AAGTCAAAAGAGC	-26580
Ankmy2 (*)	✓	AAGTCATCCTCAAATAACAAGTCA	-11147
P2 and P21			
Nr2c1		TAGTCAAAAATGC	-13001
Nr1d1		TGGGTTTGTACTT	-15611
Nr2e3		AAGTCAAAATGTGT	-3550
Rgr		GACCTTTAGACTT	-1626
Rora	✓	AAGTCAAAATGTG	-1849
Gnb3 (*)	✓	AAGTCAAAATCA	-8798