Spring 2016

Epigenetic characterization of human retina cells

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Epigenetic Characterization of Human Retina Cells

An Honors Program Project Presented to
The Faculty of the Undergraduate
College of Science and Mathematics
James Madison University

by Nicholas Robert Dunham

May 2016

Accepted by the faculty of the Department of Biology, James Madison University, in partial fulfillment of the requirements for the Honors Program.

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PUBLIC PRESENTATION

This work is accepted for presentation, in part or in full, at University Maryland, Baltimore County 18th Annual Undergraduate Symposium in the Chemical and Biological Sciences on 10/3/15, James Madison University College of Science and Math Summer Symposium on 7/30/15, and James Madison University Biosymposium on 4/15/16.
Dedication

I would like to dedicate this thesis to my family and friends for their constant support and instilling within me a desire to succeed.
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I would like to thank first and foremost Dr. Raymond Enke for accepting me as an undergraduate researcher and for the support and patience that he has given me. I would also like to thank Dr. Jon Monroe and Dr. Marta Bechtel for agreeing to be readers in this thesis. I would like to thank Christophe Langouet-Astrie, Morgan Hedden, and Annamarie Meinsen as well as other lab members, for their support. Funding for this project was provided in part by 4-VA Collaborative Research Grant, Commonwealth Health Research Board Grant, 2015 Crabtree Summer Research Scholarship, and support from the Department of Biology at James Madison University.
DNA methylation is an epigenetic modifier that modulates gene expression in plant and vertebrate genomes. The aim of this study was to characterize the role of DNA methylation in the human retina, particularly within rod and cone photoreceptor retinal neurons. Previous studies investigating DNA methylation in murine retinal cells and retina-derived human retinoblastoma immortalized cell culture lines demonstrate an inverse relationship between DNA methylation and transcriptional activity. Here, we used gene-specific bisulfite pyrosequencing analysis to measure DNA methylation in the genomes of human ocular cells in an effort to characterize the role of this important epigenetic modifier. These results can be summarized as follows: 1) human ocular tissues demonstrate tissue-specific patterns of DNA methylation on retina specific genes. These patterns demonstrate an inverse relationship with mRNA gene expression. 2) Within the human retina, cell type-specific patterns of DNA methylation are also observed between rod and cone photoreceptor neurons. These cell-specific patterns of DNA methylation demonstrate a more complex relationship with mRNA gene expression. 3) Putative Cone-rod homeobox, CRX, binding sites in rod and cone photoreceptors demonstrate differential methylation. Collectively these results demonstrate a previously undescribed role for DNA methylation in regulating gene expression in adult human retinal neurons and suggest insights into the specific mechanism of regulation.
Introduction

Blindness affects over 1.3 million Americans, while another 2.9 million Americans suffer from low vision. Major diseases involved in retinal cell death are diabetic retinopathy, retinal detachment, retinoblastoma, cone-rod dystrophy, Leber congenital amaurosis, congenital stationary night blindness, retinitis pigmentosa, age-related macular degeneration, and glaucoma. Retinitis Pigmentosa (RP) is a collection of diseases resulting in the degradation of rods in the periphery of the retina, resulting in tunnel-vision, night blindness and eventual total blindness. Age-related macular degeneration (AMD) occurs when the macula begins to degrade, resulting in blurry central vision and terminates in blindness. Glaucoma is characterized by the build-up of the vitreous humor, a fluid within the eye, resulting in damage to retinal ganglion cells due to intraocular pressure. Within the United States, over 2 million individuals over the age of 50 have AMD, while over 2.7 million U.S. citizens have glaucoma. Within Virginia alone, AMD and glaucoma affect over 110,000 individuals. With such prevalent diseases, vision research is crucial to better understand and treat human retinal issues.

The eye has several important structures required for function. Initially light passes through the cornea and the lens before striking the retina at the back of the eye (Figure 1A). The retina is a layer of highly specialized neuronal cells that lines the interior of the eye and is imperative for vision. The retina absorbs photons of light triggering a signaling cascade that is received at the visual cortex within the occipital lobe. In order for the visual cortex to receive the signal, multiple cell types must
coordinate their activities within retinal layers. The outer layer of the retina consists of rod and cone photoreceptors (PRs) (Figure 1B). An inner layer of cells consists of amacrine cells, horizontal cells, and bipolar cells. The inner layer forms post-synaptic interactions with the outer layer photoreceptors and begin the integration of signals\textsuperscript{1,3}. The ganglion cell layer receives signals from the inner layer and shuttle the synapse through the optic nerve to the visual cortex. Situated immediately behind the retina distal to the outer PR layer, is the retinal pigment epithelium (RPE). The RPE acts as a barrier between blood capillaries and retinal cells, acting as a support system, recycling vital molecules for photoreceptor function\textsuperscript{54}. The initiation of the visual signaling pathway known as phototransduction occurs within specialized cells known as photoreceptors. Photoreceptors absorb photons and convert light energy into an electrochemical signal that is relayed to other retinal cells and then ultimately to the brain through the optic nerve. Photoreceptors exist in two general classes: rods and cones. Highly sensitive rods can be activated by a single photon of light and are functional for achromatic low-light vision. Less sensitive cones allow for color and high acuity vision\textsuperscript{1}. While most mammals are dichromats, having only green and blue-sensitive cone photoreceptors, humans have evolved a red-sensitive cone and are functional trichromats\textsuperscript{1}. Photoreceptor classes are distributed unevenly throughout the human retina. Nearly all cone photoreceptors lie within a central region of the retina known as the macula. Within the central macular region of the retina exists the fovea, a 1.5 mm diameter region consisting of solely cone photoreceptors\textsuperscript{28,3,55}. Cone density falls off sharply in retinal tissue immediately adjacent to the fovea with nearly all cones being accounted for within 10 degrees of eccentricity to the fovea. This larger 3 mm
diameter region encompassing the macula is collectively an extremely cone-rich region of the human retina. The macula and fovea are clinically relevant tissues that are responsible for most of the high acuity central vision and color vision that we rely on for day-to-day functionality in the sighted world. Diseases such as AMD and cone rod dystrophy that result in degeneration of this relatively small portion of the retina, render patients legally blind\textsuperscript{1,10}. Retinal tissues surrounding the macula are collectively referred to as the peripheral retina which contains nearly all rod photoreceptors with very few cones (Figure 1C,D)\textsuperscript{28,55}. These regions of the retina are responsible for peripheral vision as well as other visual functionalities such as depth perception, detection of movement, day/night circadian rhythm and contraction of light sensitive musculature features in the posterior portion of the eye\textsuperscript{1}. Clinically relevant diseases such as retinitis pigmentosa and Leber congenital amaurosis typically cause degeneration of rod photoreceptors in the periphery. While this primary dysfunction does not dramatically affect patient’s vision, loss of rods in the periphery causes toxicity in the retina leading to secondary loss of cones in the central macula\textsuperscript{1-10}. No photoreceptors or other retinal cells exist within the optic disk, where retinal ganglion cell axons exit the intraocular space towards the visual cortex via the optic nerve.
**Figure 1.** An overview of the anatomy of the eye and retina tissues. (**A**) A schematic of the eye highlighting crucial features involved in ocular function\(^1\). (**B**) Schematic image of the retina demonstrating specialized retinal cells and layers\(^1\). (**C**) Distribution of photoreceptor density within the retina\(^{1,76}\). (**D**) A view of the human retina as seen through an ophthalmoscope\(^77\). The dark area around the central fovea represents the macula.
Photoreceptor development within the retina relies on a complex network of gene regulation controlled by the retina-specific transcription factors OTX2, CRX and NRL \(^6\). Orthodontic Homebox 2 (OTX2) is a transcription factor that regulates differentiation of RPE, bipolar cell as well as rod and cone photoreceptors from early retinal neuron precursors \(^30,31\). Cone-rod Homeobox (CRX), a paralog and direct downstream target of OTX2, is critical for further differentiation of photoreceptor precursors into fully differentiated rods and cones \(^17,32\). CRX is a transcription factor that directly regulates hundreds of genes critical for photoreceptor development, function and survival \(^17,32\). CRX has been identified as binding to hundreds of genomic regions in or adjacent to genes in the murine retina, demonstrating that CRX is a crucial factor for the expression of photoreceptor-specific genes \(^17\). Neural Retina Leucine Zipper (NRL), one of CRX’s downstream targets, encodes a transcription factor critical for rod-specific differentiation and function \(^33-36\). Photoreceptor precursors expressing NRL will suppress the cone differentiation pathway and are fated to become rods. Mice with the NRL gene knocked out fail to develop any functional rods and instead develop cone-only retinas for a brief window of postnatal life prior to the degeneration of the entire photoreceptor layer \(^34,36,37\). Studies using juvenile wild type, wt, mice with rod-rich retinas and Nrl\(-/-\) mutant mice with cone rich retinas have been exploited to determine rod and cone-specific patterns of both mRNA gene expression and transcription factor binding in the murine genome \(^17,63\).
Specialized genes explicitly for photoreceptor function include *RBP3, RHO, OPN1LW, OPN1MW, OPN1SW, PDE6B, PDE6C, ARR3, GNAT1, and GNAT2*. All of these genes are regulated by CRX and are expressed only in photoreceptor neurons⁷,¹⁷,³². Collectively, these genes participate in the phototransduction signaling cascade that results in cleavage of cAMP and closure of gated ion channels on the photoreceptor membrane. Rhodopsin, RHO, is a transmembrane G-Protein coupled receptor found exclusively within rod photoreceptors that houses a chromophore that absorbs photons of light and converts the light energy into a chemical signal⁴⁰,⁴¹. Opsin 1 (cone pigments), long-wave-sensitive, medium-wave-sensitive, and short-wave-sensitive, OPN1LW, OPN1MW and OPN1SW, are the cone-specific genes coding for the GPRC paralogs to RHO in red, green and blue cones, respectively. These opsins are sensitive to chromatic light in the long, medium and short wavelengths correlating to red, green and blue light absorption, respectively⁴²,⁴³. Phototransduction is initiated by photons of light being absorbed by chromophores gripped by the GPRC opsin proteins in the outer segments of rod and cone photoreceptors (Figure 2B). Light activated opsins undergo a conformational change and subsequent activation of opsin-associated heterotrimeric guanine nucleotide-binding proteins (G-proteins). Rod and cone-specific alpha subunits of the G-protein complex are coded for by *GNAT1* and *GNAT2* respectively⁴⁴-⁴⁶. Activated G-proteins result in subsequent activation of the phosphodiesterase 6 (PDE6) complex. Similar to the G-protein complex, rod and cone-specific PDE6 complexes are dictated by differing subunits. The PDE6 beta subunit in rods is coded for by the rod-specific gene *PDE6b* while the cone-specific beta subunit is coded for by *PDE6c*⁷⁴. The activated phosphodiesterase complex catalyzes the rapid
hydrolysis of cGMP, resulting in the closure of cGMP-gated cation channels\textsuperscript{47-49}. Closure of cGMP-gated ion channels prevents the influx of cations, resulting in hyperpolarization of the photoreceptor. The hyperpolarization then decreases the amount of glutamate at the synaptic terminal, which is processed by the post-synaptic neurons\textsuperscript{65,66}. Immediately following phototransduction the photoreceptor becomes reset. This resetting involves inactivating the opsin proteins, chromophore recycling, and reestablishment of the cation influx. Retinol-Binding Protein 3, RBP3, is involved in a process known as the visual cycle which takes spent retinoid molecules from photoreceptors and reconvert the retinoids into a useable form by the opsin protein\textsuperscript{39}. Arrestin inactivates opsin proteins by binding to phosphorylated opsin proteins. Following Arrestin binding, the opsin is no longer able to induce activation of the GNAT proteins\textsuperscript{67,68,69}. Removing the activation signal allows the photoreceptor to return to a dark-like environment, with the influx of cations returning to non-stimulated levels. Collectively, these tissue-specific genes are critical for initiating and resetting the visual perception pathway. Blinding disease-associated mutant alleles have been documented for many of these phototransduction genes\textsuperscript{10} (Table 1).
**Figure 2.** Phototransduction in the outer segments of rod and cone photoreceptors (A) Schematic depicting morphology of rods and cones. (B) Overview of the phototransduction signaling pathway within the outer segment membrane of rods and cones. Light strikes the chromophore held in the opsin protein, activating the opsin. Activated opsins in turn activate associated G-proteins (GNAT), which subsequently activate a phosphodiesterase (PDE6). Phosphodiesterase activity cleaves cyclic-GMP, cGMP, resulting in closure of cGMP-gated cation channels on photoreceptor membranes. Channel closing results in cellular hyperpolarization, and the restriction of glutamate release at photoreceptor synaptic termini. RBP3 recycles and reloads chromophore into the opsin for subsequent rounds of phototransduction. Image is adapted from Leskov et al.75
Table 1. Human retinal diseases associated with photoreceptor-specific genes investigated in this study\textsuperscript{10}.
<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Disease Name</th>
<th>Disease Type</th>
<th>Cure/Treatment</th>
</tr>
</thead>
<tbody>
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<td>Cone-rod dystrophy</td>
<td>autosomal dominant</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Leber congenital amaurosis</td>
<td>autosomal recessive/autosomal dominant</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Retinitis pigmentosa</td>
<td>autosomal dominant</td>
<td>none</td>
</tr>
<tr>
<td>RBP3</td>
<td>Retinitis pigmentosa</td>
<td>autosomal recessive</td>
<td>none</td>
</tr>
<tr>
<td>PDE6b</td>
<td>Congenital stationary night blindness</td>
<td>autosomal recessive</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Retinitis pigmentosa</td>
<td>autosomal recessive</td>
<td>none</td>
</tr>
<tr>
<td>PDE6c</td>
<td>Cone-rod dystrophy</td>
<td>autosomal recessive</td>
<td>none</td>
</tr>
<tr>
<td>RHO</td>
<td>Congenital stationary night blindness</td>
<td>autosomal dominant</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Retinitis pigmentosa</td>
<td>autosomal recessive/autosomal dominant</td>
<td>none</td>
</tr>
<tr>
<td>NRL</td>
<td>Retinitis pigmentosa</td>
<td>autosomal recessive/autosomal dominant</td>
<td>none</td>
</tr>
</tbody>
</table>
Epigenetics is the study of reversible and heritable chemical modifications to the genome that influence gene expression but do not alter the genetic code. There are two classical epigenetic categories: histone modifications and DNA methylation. Histone modification occurs on histone tails that can undergo phosphorylation, ubiquitination, methylation, acetylation, and sumoylation\textsuperscript{16,56-58}. Depending on the placement and nature of the modification, modifications can signal for transcriptional repression or transcriptional activation. Histone modifications are thought to act by remodeling local chromatin, allowing or restricting access to surrounding DNA\textsuperscript{15}. DNA methylation occurs when a methyl group undergoes enzymatic addition to the 5’ position of a cytosine in a CG dinucleotide (CpG)\textsuperscript{12,79}. CpG dinucleotides are not common throughout the human genome and instead exist largely as CpG islands, clusters of high-density CpG dinucleotides\textsuperscript{13,14}. Addition of methyl groups to the 5’ position of CpG cytosine bases in the genome are catalyzed by the DNA methyltransferase (DNMT) family of enzymes (Figure 3A). The presence of a 5-methyl cytosine (5mC) is associated with transcriptional repression in plant and vertebrate genomes by altering transcriptional machinery’s access to DNA\textsuperscript{15,7,59}. The biochemical consequences of DNA methylation are thought to be mediated by the recruitment of methyl binding domain transcriptional repressors combined with occluding the binding of transcriptional activators (Figure 3B)\textsuperscript{57-59}.
Figure 3. DNA methylation of cytosine nucleotides. (A) Brief overview of the addition of a methyl group to the 5’ position of cytosine. (B) Biochemical consequences of 5mC.
A

\[
\begin{align*}
\text{cytosine} & \quad \text{5-methyl cytosine (5mC)} \\
\end{align*}
\]

B

Transcriptional Repressor

Transcriptional Activator

5mC  5mC  5mC  5mC
A more comprehensive understanding of photoreceptor-specific gene regulation is vital for gaining novel insights into retinal function and developing novel therapeutic strategies for retinal degeneration. While transcriptional networks of retinal gene expression have been well studied, epigenetic regulation of the retina and photoreceptors has only just begun to be characterized. Previous literature has observed an inverse correlation between DNA methylation and gene expression for retina-specific genes, however, these studies have not investigated human retinal tissue and do not offer mechanistic insight to the relationship between methylation and gene expression\textsuperscript{7,37,70,71}.

More thorough characterization has been conducted on the recruitment and influence of histone modifying enzymes to regulatory regions of retina-specific genes. Previously, CRX was shown to bind near the transcriptional start site (TSS) and regulatory sequences of photoreceptor-specific genes, determining the expression of genes in rods or cones\textsuperscript{17}. Peng and Chen determined that CRX bound to proximal promoters of photoreceptor-specific genes recruits histone acetyl transferases (HATs) to local nucleosomes that mediate the addition of an acetyl group to the lysine 27 residue of histone H3 (H3K27ac). This is an epigenetic marker associated with transcriptional activation of adjacent DNA sequences in murine and human cells\textsuperscript{72}. Peng and Chen further determined that the CRX-HAT-H3K27ac complex increases the propensity of chromosomal looping, connecting distal photoreceptor-specific regulatory sequences to TSSs in both human and murine cells\textsuperscript{73}. The complex is required for both recruitment of RNA polymerase II and transcription of PR-specific genes. Collectively, these studies demonstrate that distal enhancer/regulatory sequences that lie many
thousands of nucleotides away from genes on chromosomes, are brought into close
three dimensional proximity mediated by the binding of CRX and CRX-dependent
epigenetic modification of both the distal enhancers as well as the proximal gene
promoters and coding sequence. Peng and Chen beautifully demonstrate this
phenomenon for the *RHO, OPN1MW, and OPN1SW* genes in both the mouse retina
and human retinal cell lines\(^2,72\). This mechanism of tissue-specific transcriptional
regulation is presumed to exist throughout rod and cone genomes. A recent genome-
wide study characterized CRX binding sites in murine rod and cone photoreceptors
identified thousands of CRX binding regions (CBRs) as potential locations of this unique
regulatory mechanism\(^{17}\). Though CRX is expressed in both cell types, this study
demonstrates differential binding of CRX to consensus binding motifs in rods vs cone
genomes suggesting a currently unknown mechanism for mediating differential cell-
specific patterns of CRX binding\(^{17}\). Additionally, CRX is expressed early within
photoreceptor precursors but binds to CBRs at variable points throughout development,
temporally regulating histone acetylation and subsequent transcription of photoreceptor-
specific genes\(^{72-73}\). These studies suggest an unknown mechanism mediating temporal
binding of CRX to regulatory sequences within developing photoreceptors. Though DNA
methylation in murine and immortalized human cell culture lines is associated with
transcriptional silencing of photoreceptor-specific genes, the mechanistic role for this
observation is not understood. Additionally, cell-specific patterns of DNA methylation
have only begun to be explored in human cell culture systems and have yet to be
described in actual human retinal tissue.
This aim of this study was to characterize patterns of DNA methylation in the genomes of human photoreceptors to gain mechanistic insights into this important epigenetic regulatory pathway by focusing on 3 objectives:

1) Characterize tissue-specific and photoreceptor cell type-specific patterns of differential DNA methylation using DNAs collected from rod and cone enriched post-mortem human donor eye samples,

2) Determine if mRNA transcription of photoreceptor-specific genes is inversely correlated with the presence of DNA methylation near the TSS, and

3) Computationally investigate the role of differential DNA methylation as the cell-specific and temporal regulator of CRX binding to CBRs in rod and cone genomes.

These studies demonstrate tissue and cell-specific patterns of DNA methylation in and around promoter and TSSs of photoreceptor-specific genes. Our preliminary gene expression analysis also indicates that DNA methylation is inversely correlated with mRNA gene expression of photoreceptor-specific genes. Additionally, our computational analysis suggests an inverse correlation between DNA methylation and CRX binding in rod and cone genomes. Collectively, these findings demonstrate an important role for DNA methylation in regulating human photoreceptor-specific genes.
while also suggesting insight into the cell-specific and temporal regulation of CRX-mediated activation of these genes.
Materials and Methods

Tissue Collection
De-identified human post-mortem donor eyes were procured from the National Disease Research Interchange. Limited donor information was obtained for each specimen (Table 2). Donor eye #5M was procured from the Johns Hopkins University Autopsy unit without any available donor information. Dissection of ocular tissue collection began by removing corneal tissue (Figure 4A). Corneas were snap frozen on dry ice and then ground into a fine powder. Four radial cuts were made to the remaining eyecup to prepare a retinal flat mount (Figure 4B). Either 6 mm or 3 mm biopsy punches were used to dissect macular retina for cone photoreceptor enrichment (Figure 4C). 6 mm biopsy punches were used to dissect peripheral retina for rod photoreceptor enrichment (Figure 4D). All samples were lysed in Qiagen RLT plus buffer and beta-mercaptoethanol and stored at -80°C.

Nucleic Acid Collection
Genomic DNA and total RNA were extracted simultaneously using a Qiagen Allprep Kit per manufacturer’s instructions. Briefly, lysates were disrupted using Qiashredder columns. Flow-through liquids were sequentially transferred to AllPrep DNA and RNA silica-based spin columns where genomic DNA and total RNA remained bound, washed and eluted respectively. An on column DNase treatment step was also applied to total RNA preps. Nucleic acid purity and quantity were assessed using a Synergy H1 spectrophotometer. Additionally, RNA quality were reported as a RNA Integrity Number
(RIN) was assessed for 6 samples used for transcriptome mRNA sequencing using an Agilent Bioanalyzer per the manufacturer’s instructions (Table 2).

**DNA Methylation Analysis**

200 ng of genomic DNA underwent Bisulfite Conversion (BS) using Zymo EZ DNA MethylGold Kit per manufacturer’s instructions. Briefly, sodium bisulfite mediates the attachment of a sulfite group to cytosine bases in genomic DNA followed by deamination, and desulfonation to uracil. 5mC in genomic DNA are resistant to this process. The resulting genome contains uracils in place of unmethylated cytosines while retaining 5mCs. This BS converted DNA was used as template for BS PCRs conducted in biological triplicate using gene-specific primers. BS PCRs were conducted in 30 uL reactions using Sigma-Aldrich JumpStart Taq, and biotinylated gene-specific primers. BS PCR reactions were run at optimized annealing temperatures for 44 cycles on a Bio-Rad C1000 Touch Thermal Cycler. Following BS PCR, amplicons were bound to streptavidin-conjugated beads, mixed with a sequencing primer and sequenced using a Qiagen Q24 Pyrosequencer to determine the percent methylation at each CpG dinucleotide in the BS PCR amplicon. Percent methylation at each CpG dinucleotide was averaged between biological triplicates and statistical significance between sample groups was determined using a one-tailed t-test where significance was less than 0.01 (p<0.01). Gene-specific primers used for BS PCR and pyrosequencing analysis are listed in Table 4.

**Gene Expression Analysis**
Human adult whole retina RNA sequencing (RNA-Seq) transcriptome data from Farkas et al. was reanalyzed to determine expression of candidate gene mRNAs\textsuperscript{20} (Table 3). Additionally, adult mouse rod-enriched and cone-enriched retina RNA-Seq transcriptome data from Brooks et al., was reanalyzed to determine rod or cone expression specificity of candidate genes mRNAs\textsuperscript{63} (Table 3). Total RNA fractions from three human post mortem donor macula and donor-matched peripheral retinas were submitted to the New York Genome Center for whole transcriptome mRNA sequencing analysis utilizing the Illumina NextSeq 500 sequencing platform. cDNA libraries representative of total mRNAs were constructed using Illumina TrueSeq kit according to manufacturer’s recommendations.

**Bioinformatics Analysis**

Previously published CRX binding regions (CBRs) in murine rod and cone genomes were aligned to the Human hg19 2009 genome assembly using the UCSC Genome Browser LiftOver software\textsuperscript{17,19}. Human genomic regions aligned with murine CBRs were then scanned for consensus CRX binding motifs using the MotifMap online browser and A Plasmid Editor (ApE) nucleic acid sequence editing software\textsuperscript{21,22,64}. Custom annotation data tracks were created for the Human hg19 2009 genome assembly in the UCSC Genome Browser murine CBRs alignments, CRX binding motifs within murine CBR alignments and BS PCR amplicons analyzed in our study. The browser extensible data (BED) formatted genome coordinates for these custom tracks is listed in Supplemental Table 1 (http://tinyurl.com/DunhamJmuS1). A custom UCSC track created by Farkas et al., was also used for mRNA isoform analysis of the *NRL* gene\textsuperscript{20}. 
Results

Cataloging and Procurement of Human Donor Eye Specimens

Post mortem human donor eyes procured from NDRI were photographically documented and cataloged according to donor age, race and sex as well as death to enucleation of the eye and death to delivery to the lab time intervals (Table 2). Donor eye #5M was procured without any donor information or time intervals. Dissected cornea, macula and peripheral retina were collected for nucleic acids extraction (Figure 4). Extracted DNAs and RNA met the required quality and purity threshold for subsequent DNA methylation and mRNA gene expression analysis as measured by standard spectrophotometry (data not shown). Macular and peripheral retina RNAs extracted from donor eyes #7-9 were subject to more intensive quality control using bioanalyzer analysis. These six selected RNAs has an RNA Integrity Number (RIN) between 7-10 and were therefore deemed sufficient to use for transcriptome mRNA sequencing analysis (Table 2).
Table 2. De-identified human eyes used for tissue collection within this study. RNA integrity number (RIN) was determined for samples submitted for whole transcriptome mRNA sequencing.
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<th>Sex</th>
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<th>death: delivery</th>
<th>Macula RIN#</th>
<th>Peripheral Retina RIN#</th>
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**Figure 4.** Post-mortem human eye tissue collection strategy. (A) Whole globe image of human eye. (B) Cornea tissue was dissected and collected followed by the removal of the anterior portion of the eye and creating a flat mount of the retina. The macula can be seen as the dark circle in the center of the eye. (C) Flat mount retina following 3 mm and 6 mm biopsy punches of the macula and peripheral retina respectively.
Cell and Tissue-specific Patterns of Gene Expression

Using adult human whole retinal transcriptome data from Farkas et. al, and juvenile murine whole retinal transcriptome data from Brooks et al., gene expression analysis for human retina-specific genes of interest was inferred (Table 4)\(^{20,63}\). The experiment conducted by Farkas et. al, consisted of human adult male whole retinal samples, with no methodology for separating rod and cone populations\(^{20}\). Mouse studies were conducted using wild type, wt, rod-rich whole retina as well as cone-rich whole retinas from Nrl-/− knockout mice to determine differential expression of rod and cone-specific genes\(^{7}\). Rod and cone-enriched total RNAs from human donor macula and peripheral retinas collected in this study have been analyzed for RNA integrity and submitted for transcriptome mRNA sequencing analysis (Table 2).
Table 3. Reexamined mRNA gene expression analysis of previously published retina-specific genes in human and mouse. 20, 63.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Expressed in Adult Human Retina&lt;sup&gt;20&lt;/sup&gt;</th>
<th>Expression Pattern in Mouse Retina&lt;sup&gt;63&lt;/sup&gt;</th>
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<tr>
<td>Long Interspersed Nuclear Element 1</td>
<td>LINE1</td>
<td>no</td>
<td>none</td>
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<tr>
<td>Paired Box 6</td>
<td>PAX6</td>
<td>yes</td>
<td>rods and cones</td>
</tr>
<tr>
<td>Cone-Rod Homeobox</td>
<td>CRX</td>
<td>yes</td>
<td>rods and cones</td>
</tr>
<tr>
<td>Retinol Binding Protein 3</td>
<td>RBP3</td>
<td>yes</td>
<td>rods and cones</td>
</tr>
<tr>
<td>Neural Retina Leucine Zipper</td>
<td>NRL isoform #1</td>
<td>yes</td>
<td>rods</td>
</tr>
<tr>
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<td>NRL isoform #2</td>
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<td>none</td>
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<td>Phosphodiesterase 6B, CGMP-specific, rod, beta</td>
<td>PDE6b</td>
<td>yes</td>
<td>rods</td>
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<tr>
<td>Recoverin</td>
<td>RCVRN</td>
<td>yes</td>
<td>rods</td>
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<tr>
<td>Rhodopsin</td>
<td>RHO</td>
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<td>yes</td>
<td>cones</td>
</tr>
<tr>
<td>Arrestin 3, Retinal (X-Arrestin)</td>
<td>ARR3</td>
<td>yes</td>
<td>cones</td>
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Table 4. Primers used for bisulfite PCR and pyrosequencing analysis. The presence of a BIO denotes 5' biotinylation modification of oligonucleotide.
<table>
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<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>region analyzed</th>
<th>PCR Annealing temp (˚C)</th>
<th>application</th>
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<td>CRX promoter</td>
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<td>58</td>
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<td>hCrx-R1-Bio</td>
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<td></td>
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<td>hCrx-seq1</td>
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<tr>
<td>hNrl F2</td>
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<td>NRL #2 long isoform promoter</td>
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<td>54-64</td>
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<td>hNrl R2-Bio</td>
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<td>54-56</td>
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**Tissue-specific Patterns of DNA Methylation Within Photoreceptor-specific Genes**

We first sought to determine if retina-specific patterns of DNA methylation exist near the transcriptional start sites, TSS, of photoreceptor genes. BS pyrosequencing analysis was conducted for the photoreceptor genes RHO, RCVRN, PDE6b, PDE6c, NRL, CRX, and RBP3 as well as the ocular-specific gene PAX6. These analyses revealed that levels of DNA methylation adjacent to the TSS of the RHO, RCVRN, PDE6b, NRL, CRX, and RBP3 genes are consistently lower in peripheral retina than in the corneal cells (Figure 5; red vs black lines). In contrast, PAX6 which is expressed in both retina and cornea, exhibited low methylation in all samples assayed (Figure 5B, black vs red lines). **LINE1**, a high copy retrotransposon that is densely methylated and constitutively transcriptionally repressed in all somatic human tissues was used as control loci to demonstrate similar levels of global DNA methylation in retina and cornea genomes60 (Figure 5A). Collectively, these data demonstrate an inverse correlation between DNA methylation and mRNA gene expression in adult human retinal and corneal tissues, similar to previously published observations in mouse retinal tissues and human cell culture models (Table 3).

**Rod and Cone-specific Patterns of DNA Methylation Within Photoreceptor-specific Genes**

Previous reports in the literature describe rod and cone-specific patterns of DNA methylation on photoreceptor-specific genes using rod-enriched retinal DNAs isolated
from wt mice and cone-enriched retinal DNAs isolated from Nrl−/− mice. We next sought to determine if these cell-specific patterns of DNA methylation also exist between human rod and cone photoreceptor neurons. Unlike mice, the human retina has a central macula, an anatomical feature that houses the vast majority of cone photoreceptors with very few rod photoreceptors (Figure 1C). Separately sampling the macular retina and the peripheral retina allowed us to enrich for cone and rod photoreceptors respectively (Figure 4C). The clinically described macula is a 5.5 mm diameter region around the central fovea that extends to 10˚ of eccentricity within the retina. A smaller region centered on the fovea including the adjacent parafovea combine to make up a 2.5 mm diameter region that extends to 7˚ of eccentricity within the retina (Figure 1C+D). We used either 6 mm or 3 mm biopsy punches to collect macular samples from human donor eyes as well as 6 mm biopsy punches of adjacent peripheral retina to characterize cell-specific patterns of DNA methylation in rod-enriched versus cone-enriched genomes (Figure 4C).

BS pyrosequencing analysis of these DNAs demonstrated several interesting cell-specific patterns of DNA methylation (Figure 5). RHO, RCVRN, NRL and PDE6b are genes known to be expressed exclusively in rod but not cone photoreceptors. Each of these genes demonstrated reduced DNA methylation in peripheral retina compared to macular retina (Figure 5C,D,F,I; red vs green and blue lines). Interestingly, RBP3 and CRX are genes that are known to be expressed in both rod and cone photoreceptors, however, both of these genes also demonstrated reduced DNA methylation in peripheral retina compared to macular retina as well (Figure 5G,H; red vs green and blue lines). PDE6c is expressed exclusively in cone and not rod photoreceptors,
however, high levels of DNA methylation were present in both macular and peripheral retina DNAs (Figure 5J; red vs green and blue lines, significance is shown as p<0.01 between 3 mm macula and peripheral retina). Collectively these data demonstrate rod and cone-specific patterns of DNA methylation in human photoreceptors. However, the observed methylation patterns are more complex than anticipated and do not always demonstrate an inverse correlate with mRNA gene expression.

Initially, the difference in DNA methylation between 6 mm macula and peripheral retina showed a differential trend but not a statistically significant difference. Following a change in methodology to collect 3 mm macula, differential DNA methylation was found to be statistically significance between macula and peripheral retinal samples (Figure 5C-D, F-H; red vs blue lines p<0.01). This finding suggests that sampling of the 3 mm region encompassing the human fovea and parafovea is sufficient for studies requiring cone enrichment.

**Analysis of Epigenetic Regulation of Cryptic Transcriptional Start Site Usage**

UCSC Genome Browser analysis of the *NRL* gene demonstrates that multiple *NRL* isoforms are expressed. RNA sequencing transcriptome data were used to determine that the longer *NRL* isoform (isoform #2) is predominantly expressed in the adult retina (Figure 6D). The Y79 immortalized cell culture line is derived from retinoblastoma tissue and has some molecular characteristics of photoreceptors. In addition to the longer canonical isoform, a shorter isoform (isoform #1) also accumulates in Y79 cells and is the result of a cryptic TSS that exists in the canonical intronic 2 sequence. In order to determine if *NRL*’s usage of the cryptic alternative
TSS is regulated by DNA methylation, BS pyrosequencing primers were designed to assay both the canonical and short isoform TSS upstream regions (Figure 6D). Unlike the canonical *NRL* TSS, the short *NRL* isoform TSS upstream region is nearly devoid of DNA methylation in all retinal and corneal tissues (Figure 5E+F). These data suggest that DNA methylation is not required for silencing the cryptic promoter in adult human retina.
**Figure 5.** Quantitative bisulfite pyrosequencing analysis of Human DNA methylation at CpG sites relative to the transcriptional start site of (A) LINE1, (B) PAX6, (C) RHO, (D) RCVRN, (E) NRL isoform 1, (F) NRL isoform 2, (G) RBP3, (H) CRX, (I) PDE6b, and (J) PDE6c. Data are presented as %5mC at indicated genomic positions. Error bars represent standard error of the mean between three biological replicates of each sample. Statistical significance between 3 mm macula and peripheral retina determined by t-test with a p<0.01 is denoted by an asterisk (*).
Computational Analysis of Functional CRX Binding Sites within Human Photoreceptors

Our experimental analysis of the human macular retina, peripheral retina, and cornea demonstrates both tissue and cell-specific patterns of DNA methylation near the TSS of photoreceptor-specific genes. In some but not all cases, these patterns of DNA methylation are inversely correlated with mRNA gene expression. This observation suggests a functional role for DNA methylation in blocking transcription machinery at these loci (Figure 3B). However, the mechanism for this blockage remains uncharacterized. A commonality all of the above loci share is that they are transcriptionally regulated by CRX during retina development in a temporal and cell-specific manner\textsuperscript{2,17,72,73}. This observation led us to question whether DNA methylation itself may be the unknown factor regulating temporal and cell-specific binding of CRX to regulatory regions within photoreceptor genomes. To test this hypothesis, we used a computational approach to align genome-wide CRX binding regions (CBRs) in mouse rod and cone photoreceptors experimentally determined by Corbo et. al, to the human hg19 2009 genome assembly (Figure 6)\textsuperscript{17}. To further assess the functionality of these regions, aligned CBRs mapping to our candidate genes of interest were searched for short consensus CRX binding motifs as well as sequence conservation among 100 vertebrate species (Figure 6). This computational analysis demonstrates a strong correlation between aligned mouse CBRs, endogenous human CRX binding motifs and sequence conservation suggesting these sites are putative functional CRX binding sites in human photoreceptors. We next mapped BS PCR amplicons analyzed in this study to the human genome assembly and found overlap of our regions of methylation analysis.
with putative functional CRX binding sites in the CRX, RBP3, RHO, PDE6B, NRL, and RCVRN loci (Figure 6B-G). Notably, the BS amplicon for PDE6C analyzed in this study lies just outside of a putative functional CRX binding site (Figure 6H). Collectively, these data suggest a potential role for differential DNA methylation regulating the accessibility of CRX binding to consensus motifs.
**Figure 6.** UCSC Genome Browser views of candidate genes in the human hg19 genome assembly. (A) Ocular-specific gene *PAX6*. Retina-specific genes (B) *CRX* and (C) *RPB3*. Rod-specific genes (D) *RHO*, (E) *PDE6b*, (F) *NRL* and (G) *RCVRN*. Cone-specific gene *PDE6c* (H). All genes are oriented with the transcriptional start site on the left. From top to bottom, data tracks display 1) human hg19 genome coordinates, 2) Mouse Rod CBR alignments, 3) Mouse Cone CBR alignment, 4) Endogenous human CRX binding motifs, 5) Vertebrate conservation, 6) BS PCR amplicons, and annotated genes and isoforms. The NRL locus also has Human Adult Retina RNA sequencing data displayed as determined by Farkas et al.,20.
**Discussion**

DNA methylation is an epigenetic modifier that integrates with histone tail epigenetic modifications to modulate gene expression in plant and vertebrate genomes. The aim of this study was to characterize the role of DNA methylation in the human retina, particularly in the genomes of rod and cone photoreceptor retinal neurons. Previous studies investigating DNA methylation in murine retinal cells and retina-derived human retinoblastoma immortalized cell culture lines often demonstrated an inverse relationship between DNA methylation and transcriptional activity\(^7,80\). However, this relationship in actual human retinal tissue had yet to be characterized. Furthermore, a mechanism explaining this phenomenon has not been described to date. In the current study, we used gene-specific bisulfite pyrosequencing analysis to measure DNA methylation in the genomes of human ocular cells in an effort to characterize the role of this important epigenetic modifier. These results can be summarized as follows: 1) human ocular tissues demonstrate tissue-specific patterns of DNA methylation on retina specific genes. These patterns of DNA methylation demonstrate an inverse correlation with mRNA gene expression. 2) Within the human retina, cell type-specific patterns of DNA methylation are also observed between rod and cone photoreceptor neurons. These cell-specific patterns of DNA methylation demonstrate a more complex relationship with mRNA gene expression. 3) Putative CRX binding sites in the genome of rod and cone photoreceptors demonstrate differential methylation. Collectively these
results demonstrate a previously undescribed role for DNA methylation in regulating
gene expression in adult human retinal neurons and suggest insights into the specific
mechanism of regulation.

Bisulfite pyrosequencing analysis of human ocular tissues demonstrated that
although global levels of DNA methylation are similar in the genomes of cornea and
retinal cells (Figure 5A), many of the retina-specific genes examined in this study exhibit
tissue-specific patterns of DNA methylation (Figure 5C-D, F-I). These genes are
transcriptionally active in the retina but not in other tissues (Table 3). These
observations support the hypothesis that an inverse relationship between gene
expression and DNA methylation exists for retina-specific genes similar to previously
characterized observations in mouse retinal tissues and immortalized retina-derived
human cell culture lines. PAX6, a gene expressed in both retina and cornea,
demonstrates low levels of methylation in both tissues (Table3, Figure 5B). Further
experimentation is required to determine if ocular-specific patterns of DNA methylation
exist within human tissues for PAX6 and other ocular-specific genes.

Bisulfite pyrosequencing analysis of rod and cone-enriched human retinal DNAs
demonstrated that although global levels of DNA methylation are similar in the genomes
of photoreceptor neurons (Figure 5A) many of the retina-specific genes examined in this
study exhibit cell type-specific patterns of DNA methylation (Figure 5C-D, F-I). This is a
phenomenon that has previously been observed in murine retinal genomes using the
rod rich wt mouse retina and the Nrl-/- genetic knockout line as a method of collecting
cone-enriched retinal DNAs. To recapitulate these studies in the human retina, we
experimentally determined the optimal region of the adult human macula to collect for
analysis of rod and cone-specific patterns of DNA methylation. Initially, 6 mm biopsy punches encompassing the clinically described cone-rich macula extending to 10° of eccentricity within the retina were sampled and compared to adjacent 6 mm rod-rich peripheral retina samples from the same donor eyes (Figure 1C+D). Pyrosequencing analysis of these tissues demonstrated a trend towards rod and cone-specific patterns of DNA methylation (Figure 5C-D, F-I red vs green lines; p>0.01). Subsequent samples were collected using 3 mm biopsy punches encompassing only the central fovea and adjacent parafovea limited to 7° of eccentricity within the retina (Figure 1C+D). Pyrosequencing analysis of these DNAs demonstrated more pronounced and statistically significant cell-specific patterns of DNA methylation (Figure 5C-D, F-I red vs blue lines; p<0.01). It should be noted that although DNAs analyzed in these experiments are enriched for either rod or cone photoreceptors, populations of other retinal cell types exist in these preparations as well. Future studies employing more rigorous methodologies for specifically collecting rod and cone-specific photoreceptors, such as laser capture microdissection and affinity purification, will be critical for fully understanding photoreceptor-specific patterns of DNA methylation.

The relationship between rod and cone photoreceptor-specific patterns of DNA methylation and mRNA expression was complex. A clear inverse correlation between DNA methylation and mRNA expression was observed for a subset of genes analyzed in this study, particularly the rod-specific genes RHO, RCVRN, NRL, and PDE6B (Table 3; Figure 5C-D,F,I, red vs blue lines). A similar trend was observed for CRX and RBP3, genes expressed in both rod and cone photoreceptors, however slightly more methylation was observed at these loci in cone-rich macular samples (Table 3; Figure
While these studies cannot rule out the possibility of rod and cone-specific patterns of methylation, a more likely explanation is that the differing methylation patterns may reflect the composition of retinal cells that make up the peripheral and macular retina samples collected in this study. More refined tissue collection methodologies such as those described above will be useful for teasing out this difference.

Somewhat confounding was the observation that *PDE6C* demonstrated high levels of methylation in both peripheral and macular DNAs despite being expressed exclusively in cone photoreceptors (Table 3; Figure 5I, red vs blue lines). This observation seems to contradict the hypothesis that presence of DNA methylation near the TSSs of genes serve as molecular blockades to transcriptional coactivators and suggests that perhaps the specific location of DNA methylation is a critical component of transcriptional regulation. Given that all photoreceptor-specific genes analyzed in this study are known to be regulated by CRX, we investigated the BS PCR amplicons used in pyrosequencing analysis for the presence of putative functional CRX binding motifs (Figure 6). These computational analyses demonstrated an overlap of differentially methylated regions (DMRs) and conserved putative functional CRX binding motifs in the *RHO, RCVRN, RBP3, CRX*, and *PDE6B* loci (Figure 5C-D,G-I; Figure 6B-E,G). Though no DMRs were found at the cone-specific *PDE6C* locus, the BS PCR amplicon used to assay this region lies adjacent to but does not encompass a putative functional CRX binding motif (Figure 5J; Figure 6H). These observations support a more refined hypothesis that DNA methylation within CRX binding motifs is inversely correlated with transcription of nearby genes. Given the previously described role for CRX-mediated
epigenetic regulation of photoreceptor-specific genes in the human and murine retina\textsuperscript{72-73}, this would explain a previously undefined role for DNA methylation in controlling the initiation of this regulatory pathway. Specifically, complex patterns of photoreceptor gene expression may be controlled by differential methylation of CRX binding motifs modulating their accessibility to CRX binding and subsequently recruitment of histone acetyltransferase enzymes. Further epigenetic analysis of DNA methylation within putative functional CRX binding motifs of \textit{PDE6C} and other cone-specific genes will help test this model. Biochemical analysis of in vitro CRX binding to methylated and unmethylated DNA oligonucleotides will also be a useful future experiment to test this hypothesis.

DNA methylation is known to play an important role in regulating the initiation of transcription of tissue-specific genes from canonical promoters in several tissues including the retina\textsuperscript{7,80}. Much less is known about the role that DNA methylation plays in modulating alternative transcriptional isoforms from cryptic promoters. Analysis of the \textit{NRL} locus demonstrates that DNA methylation is associated with tissue-specific transcriptional regulation of the canonical upstream promoter region. Methylation however, does not seem to be required to silence a non-canonical transcript variant initiating from a cryptic downstream promoter (Figure 5E-F; 6F). Analysis of additional retina-specific loci will be conducted to determine if there is a relationship between DNA methylation and cryptic TSS isoforms. We are currently awaiting pending mRNA-Seq transcriptome data collected from human donor 3 mm macula and 6 mm peripheral retina total RNAs. Bioinformatics analysis of these data will be used to in part to identify
rod and cone-enriched isoforms originating from alternative cryptic promoters for follow-up DNA methylation analysis.

In summary, DNA methylation is an important epigenetic modifier in the genomes of human retinal neurons. Methylation is associated with retina-specific and photoreceptor-specific gene expression patterns in the human retina similar to previous observations in the murine and retina-derived cell line genomes. The full extent of DNA methylation’s role in regulating gene expression remains to be determined, however, future studies investigating differential methylation specifically in CRX and other retina-specific transcription factor binding motifs may lead to further insights in this area. We are hopeful that further analyses of this mechanism will provide insights into epigenetic control in the normal retina as well as in diseased states, giving potential for novel therapeutic targets of retinal disease.
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   [http://biologylabs.utah.edu/jorgensen/wayned/ape/](http://biologylabs.utah.edu/jorgensen/wayned/ape/)


