CASE WESTERN RESERVE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

We hereby approve the thesis/dissertation of

______________________________
DEBARSHI MUSTAFI

candidate for the ______ Ph.D ____________ degree *

(signed) ______________________
Dr. Johannes Von Lintig
(chair of the committee)

______________________________
Dr. Krzysztof Palczewski

______________________________
Dr. Robert A. Bonomo

______________________________
Dr. George Dubyak

______________________________
Dr. Andreas Engel

______________________________
Dr. Vera Moiseenkova-Bell

______________________________
Dr. Jonathan E. Sears

(date) ______ May 31, 2013

*We also certify that written approval has been obtained for any proprietary material contained therein
TABLE OF CONTENTS

LIST OF TABLES ...........................................................................................................viii

LIST OF FIGURES ..........................................................................................................x

ACKNOWLEDGMENTS .................................................................................................xiv

LIST OF ABBREVIATIONS ..............................................................................................xvi

ABSTRACT .........................................................................................................................1

CHAPTER 1: THE GENOTYPE TO PHENOTYPE QUESTION

UNDERLYING VISION .........................................................................................................2

1.1 The eye

1.1.1 Features of the eye across species .................................................................3

1.1.2 The retinal layer and the role of rod and cone photoreceptors .................5

1.1.3 The importance of cone cells in the context of retinal disease ..........6

1.2 Cone phototransduction signaling

1.2.1 The first steps in visual response by cones .........................................7

1.2.2 Structural dynamics of cone opsins during phototransduction ..........9

1.3 Evolutionary dynamics of the eye

1.3.1 Evolution of retinal circuitry ..............................................................12

1.3.2 Evolution of cone photoreceptors .................................................13

1.4 Structural features of cone photoreceptors

1.4.1 Cone disc renewal .................................................................16

1.4.2 Cone disc morphogenesis ..................................................19

1.5 Mammalian models to better elucidate cone cell structure and function
1.5.1 Mouse models to study cone cells……………………………………………..22
1.5.2 Other mammalian rodent species for study of cone cells…………………..23

1.6 The genotypic to phenotype connection in the retina in the context of disease

1.6.1 High resolution phenotypic methods to reveal retinal architecture……….24
1.6.2 High throughput sequencing to reveal global gene expression patterns
in the retina………………………………………………………………………25

1.7 Project approach………………………………………………………………......26

Figures……………………………………………………………………………28

CHAPTER 2: PROGRESSIVE DEGENERATION IN MONOGENIC
ENHANCED S-CONE SYNDROME IS DRIVEN BY ABBERANT
RETINAL HOMEOSTASIS……………………………………………………………38

2.1 The monogenic disease enhanced S-cone syndrome

2.1.1 The human condition and the corresponding mouse model of disease……39

2.2 Rationale and methodology to elucidate the degenerative component of ESCS

2.2.1 Rationale for research approach……………………………………………41
2.2.2 Materials and methods……………………………………………………..42

2.3 Results

2.3.1 Phenotypic features of human ESCS patients and the relationship
of these features to the Nrl<sup>-/-</sup> mouse model………………………………56
2.3.2 Transcriptome analysis by Illumina based RNA-Seq of retinas from
Wt and Nrl<sup>-/-</sup> mice………………………………………………………………..59
2.3.3 Verification of sequencing data by RT-PCR…………………………………60
2.3.4 Characterization of differentially expressed transcripts…………………...61
2.3.5 Disrupted ESCS retinal architecture and patchy loss of photoreceptors….63
2.3.6 ESCS photoreceptors exhibit abnormal accumulations of material……..64
2.3.7 Aberrant distribution of disc membranes influences abnormal packing
architecture of ESCS photoreceptors…………………………………………….65
2.3.8 Evidence for aberrant phagocytosis in ESCS disease……………………..67
2.3.9 ESCS phenotype attributed to photoreceptor abnormalities rather
than a RPE defect……………………………………………………………………69

2.4 Discussion and conclusions…………………………………………………….70

Tables…………………………………………………………………………………………77

Figures…………………………………………………………………………………………82

CHAPTER 3: DIFFERENTIAL BACKGROUND GENETIC NETWORKS

DRIVE MULTIGENIC AGE-RELATED RETINAL DEGENERATION………102

3.1 The multi-genic etiology of age-related retinal degeneration (ARD)
   3.1.1 Age-related pathology in the eye and the genetic contributions
       Influencing disease…………………………………………………………….103

3.2 Rationale and methodology to multi-genic contributions driving ARD
   3.2.1 Rationale for research approach………………………………………….104
   3.2.2 Materials and methods……………………………………………………105

3.3 Results
   3.3.1 A/J genetic background mice undergo pronounced age–related
       retinal degeneration…………………………………………………………109
3.3.2 ARD in A/J mice is accompanied by inflammatory cell infiltration and RPE cell pathology…………………………………………………………110
3.3.3 RNA−Seq reveals differential genetic background contributions to the transcriptome……………………………………………………………..112
3.3.4 Pathway analysis highlights inflammatory priming coupled with impaired retinal homeostatic cellular pathways in 1−month−old A/J mice before retinal pathology is evident…………………………………………..114
3.3.5 Inflammatory priming in A/J retina is exacerbated with age………………115
3.3.6 Marginally−expressed retinal homeostasis proteins exhibit abnormal RPE localization in A/J mice………………………………………………...116

3.4 Discussion and conclusions………………………………………………………..117

Tables……………………………………………………………………………………124
Figures……………………………………………………………………………………129

CHAPTER 4: THE ROLE OF NON-CODING RNAs IN VISUAL FUNCTION……………………………………………………………………………156

4.1 Delineating roles of long intergenic non-coding RNAs in the adult retina

4.1.1 Long intergenic non-coding RNAs and their possible physiological roles…………………………………………………………………………………………157

4.2 Rationale and methodology to reveal evolutionary conservation of lincRNAs across species as a determinant of functional preservation in the eye

4.2.1 Rationale for research approach……………………………………………..158
4.2.2 Materials and methods………………………………………………………..159
4.3 Results

4.3.1 RNA–Seq identifies eye lincRNAs that exhibit sequence conservation in mammals and those that exhibit conservation in the human retina and macular region.................................................................164

4.3.2 Tissue and eye compartment expression of conserved lincRNAs.............166

4.3.3 Expression of some conserved lincRNAs is localized to specific retinal layers.................................................................................................................167

4.3.4 Genetic loci and in silico analyses of promoter motifs highlight possible roles of lincRNAs in adult retinal homeostasis.............................................168

4.4 Discussion and conclusions.................................................................169

Tables.............................................................................................................174

Figures..........................................................................................................181

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS.......................188

REFERENCES..............................................................................................199
LIST OF TABLES

**Table 1**: GO term breakdown of transcript reads across different RNA-Seq experiments with Wt and Nrl⁻/⁻ tissues…………………………………………………………77

**Table 2**: Fold changes of selected transcripts in Nrl⁻/⁻ relative to Wt tissue across different experiments…………………………………………………………………….78

**Table 3**: Transcript levels of visual cycle proteins in Nrl⁻/⁻ relative to Wt tissue across different RNA-Seq runs………………………………………………………….79

**Table 4**: Transcript levels (in FPKM) of putative phagocytosis proteins in Nrl⁻/⁻ relative to Wt tissue across different RNA-Seq runs……………………………………81

**Table 5**: GoTerm breakdown of transcript reads across different RNA-Seq experiments with from 1-month-old A/J, BALB/c and B6 mice whole eye tissue………124

**Table 6**: Differential expression profile of genes in the A/J and B6 mouse eye and SNP analysis of differentially expressed genes……………………………..……..125

**Table 7**: Transcript reads (FPKM) of selected genes from A/J, BALB/c, and B6 mice as well as from Long-Evans rat and Nile rat eyes…………………………………127

**Table 8**: Profile of 18 conserved lincRNAs in the eye across species………………174

**Table 9**: Profile of 18 conserved lincRNAs in the retina across species………………175

**Table 10**: Transcript abundance (FPKM) in the eye and retina of B6 mice…………..176

**Table 11**: Profile of 18 conserved lincRNAs in 4 biological replicates of macaque macula tissue…………………………………………………………………177

**Table 12**: Semi-quantitative RT-PCR of conserved lincRNAs in organs and eye compartments of 1 month old C57BL/6 mice………………………………….…….178
Table 13: Semi-quantitative RT-PCR of conserved lincRNAs in mice with different retinal environments……………………………………………………………………179

Table 14: In silico promoter analysis of conserved lincRNAs reveals binding sites for transcription factors that drive retinal processes………………………………………………………180
LIST OF FIGURES

**Figure 1:** Differences in photoreceptors and their arrangement in the retina..........28

**Figure 2:** Distribution of photoreceptors in the eye...........................................29

**Figure 3:** The steps in cone phototransduction.....................................................30

**Figure 4:** Structure and renewal of rod and cone discs ......................................31

**Figure 5:** Homology model of S-cone (blue) ops...in ...........................................32

**Figure 6:** Evolution of photoreceptors .................................................................33

**Figure 7:** Schematics of retinal sample preparation and FIB-SEM based experimentation...........................................................................................................34

**Figure 8:** Experimental set-up for photoreceptor observation with FIB-SEM technology............................................................................................................35

**Figure 9:** Schematic of SBF-SEM experimentation................................................36

**Figure 10:** High-throughput sequencing reveals global gene expression patterns........37

**Figure 11:** Key features of human ESCS disease and the *Nrl−/−* mouse model ..........82

**Figure 12:** ESCS photoreceptors of *Nrl−/−* mice display aberrant packing...........84

**Figure 13:** Reproducibility of murine eye RNA-Seq experiments and globally differentially expressed genes detected between Wt and *Nrl−/−* whole eyes.................85

**Figure 14:** RNA-Seq of Wt and *Nrl−/−* retinas reveals new differentially expressed genes arising from transcriptional mis-regulation.........................................................87

**Figure 15:** ESCS photoreceptors of *Nrl−/−* mice display aberrant packing and OS morphology caused by build-up of material in OS heads and aberrant photoreceptor phagocytosis..................................................................................89

**Figure 16:** TEM confirms that older *Nrl−/−* mouse retinas display full course of
impaired phagocytotic degeneration

Figure 17: Layers of neatly stacked rod photoreceptors in Wt mouse retina

Figure 18: Internal architecture of Wt mouse rod photoreceptors

Figure 19: Internal architecture of Nrl⁻/⁻ photoreceptors

Figure 20: Three-dimensional reconstructions from FIB-SEM data reveal packing architecture and internal elements of photoreceptors and the changes that accompany ESCS disease

Figure 21: SBF-SEM allows visualization of impaired phagocytosis present in ESCS retinal degeneration

Figure 22: Absent phagosome staining at the photoreceptor-RPE interface confirms impaired phagocytosis in Nrl⁻/⁻ mice

Figure 23: Wt and Nrl⁻/⁻ mouse RPE phagocytose both Wt and Nrl⁻/⁻ photoreceptor OS membranes

Figure 24: Transcriptional mis-regulation causes precocious development of cone-like cells in the Nrl⁻/⁻ retina, which are then maintained by transcriptional networks that alter key homeostatic processes

Figure 25: A/J mice display a pronounced age-dependent decline in vision

Figure 26: A/J and B6 mouse eyes exhibit different retinoid content profiles

Figure 27: Age-related degenerative changes in eyes of A/J mice are independent of lighting conditions

Figure 28: Mild progression of age-dependent retinal dysfunction in BALB/c mice

Figure 29: Increased retinal autofluorescence in A/J mice with age relates to
inflammatory changes and immune cell infiltration……………………………………..136

**Figure 30:** Significant pathological changes are apparent in RPE cells of A/J mice before measurable visual decline……………………………………………………………..137

**Figure 31:** Increased autofluorescence in BALB/c mice with age occurs without obvious pathological changes in the RPE……………………………………………………139

**Figure 32:** Genetic panel study reveals no significant phenotypic changes in B6 mice with single A/J chromosome substitutions………………………………………………141

**Figure 33:** RNA−Seq of biological replicates of 1-month old eye tissue from A/J, BALB/c and B6 mice reveals high reproducibility of individual background runs…………………………………………………………………………142

**Figure 34:** RNA−Seq data are verified by RT−PCR and immunoblotting…………………………..144

**Figure 35:** RNA−Seq of three individual biological replicates of 1-month-old A/J, BALB/c and B6 mouse eyes reveals differential transcriptome profiles……………………145

**Figure 36:** Key SNPs are identified by RNA-Seq…………………………………………………………..147

**Figure 37:** Pathway analysis of RNA−Seq differential expression profiles reveals age−related inflammatory priming in eyes of A/J mice……………………………………149

**Figure 38:** Homeostatic processing genes with decreased expression in A/J mice display protein expression in RPE and photoreceptor compartments of the retina……..151

**Figure 39:** *In vivo* ROS generation is detected in the RPE of aged A/J mice…………………..153

**Figure 40:** Inadequate protection by the RPE from stress drives the retina from an inflammatory−primed state to a chronic disease state……………………………………155

**Figure 41:** RNA−Seq of eye tissue from rodents and higher order mammals reveals sequence conserved lincRNAs………………………………………………………181
**Figure 42:** LincRNAs displaying conservation in the macular region of the retina highlight their potential role in higher order visual processing………………………...183

**Figure 43:** LincRNAs display spatially restricted expression in adult B6 mouse organs and eye compartments………………………………………………………………184

**Figure 44:** Enrichment profiles in mouse models with varying photoreceptor populations and *in situ* hybridization reveal cellular localization of lincRNAs to specific retinal layers………………………………………………………………185

**Figure 45:** Location in the mouse genome and promoter analysis highlight possible roles for lincRNAs in retinal homeostasis……………………………………187
ACKNOWLEDGEMENTS

First and foremost I would like to thank my thesis advisor, Dr. Krzysztof Palczewski, for his guidance throughout my time in the lab. Dr. Palczewski challenged me from the first day I entered the lab and instilled in me a strong work ethic, which he personified. Most of all, I am grateful for his constant support and his willingness to sit and discuss data for a few minutes or hours as the mood struck. He has taught me lessons I will carry throughout my research career and because of him I will forever fondly remember my graduate school experience.

I was very fortunate to be surrounded by exceptional scientists in the Palczewski laboratory who were always willing to help and discuss ideas. Dr. Brian Kevany, a postdoctoral fellow who joined the lab as the same time as me, was an invaluable resource for me. He not only taught me many scientific techniques but was always willing to discuss ideas and how to overcome difficult steps of a project. I also appreciated the scientific discussions with Dr. Marcin Golczak and his guidance throughout my time in the lab. Also, our lab manager, David Peck, was instrumental in keeping the lab running smoothly, and most of all, was brave enough to help me handle Nile rats and ground squirrels to accomplish my thesis work.

I am also grateful for the excellent collaborations that allowed many aspects of the work to progress. I would like to thank Dr. Andreas Engel, who was like a second thesis advisor to me, and taught me the finer points of many microscopy techniques. Dr. Tadao Maeda was instrumental in teaching me many aspects of mouse physiology and genetics in the beginning of my training. Finally, Dr. Mark Adams, while he was here in
the Genetics department, was an excellent mentor regarding deep sequencing technologies and was always willing to troubleshoot in the beginning when we were trying to get our tissues to work with the methodology. Without his help I am certain this thesis work would not have progressed as it has.

I also would like to thank my thesis committee members, Drs. Johannes von Lintig, Robert Bonomo, George Dubyak, and Vera Moiseenvoka-Bell for their helpful advice and willingness to adapt as my thesis project changed over time. I also would like to thank Drs. Andreas Engel and Jonathan Sears for taking the time to meet regarding my thesis work.

I also would like to express my gratitude to the medical scientist training program for giving me such an opportunity to pursue this career and for the enormous support from the directors, Drs. Harding and Dubyak, and Kathy, Bart and Jane in the office for making the medical and research worlds never seem that disconnected.

Finally, I am grateful to the funding from the National Institutes of Health via the MSTP and VSTP training grants as well as funding from the NEI to Dr. Palczewski that has allowed me to carry out such an interesting research project.

At the end of it all, I want to thank those two people who have been with me since the first day, my mom and dad. I cannot express how grateful I am for their continued love and support as I have found my way, with bumps along the road. As a child I was always captivated by their passion for science as PhD scientists, and with the culmination of my work here, the biggest honor for me is to join them in that regard.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>4',6-diamidino-2-phenylindole</td>
<td>DAPI</td>
</tr>
<tr>
<td>Age–related macular degeneration</td>
<td>AMD</td>
</tr>
<tr>
<td>Age-related retinal degeneration</td>
<td>ARD</td>
</tr>
<tr>
<td>Autofluorescence</td>
<td>AF</td>
</tr>
<tr>
<td>Basal evagination</td>
<td>BE</td>
</tr>
<tr>
<td>Basic helix loop helix</td>
<td>bHLH</td>
</tr>
<tr>
<td>C57BL/6J-B6</td>
<td></td>
</tr>
<tr>
<td>Case Western Reserve University</td>
<td>CWRU</td>
</tr>
<tr>
<td>Chromosome substituted strain</td>
<td>CSS</td>
</tr>
<tr>
<td>Complement Factor H</td>
<td>CFH</td>
</tr>
<tr>
<td>Complementary DNA</td>
<td>cDNA</td>
</tr>
<tr>
<td>Cone outer segment</td>
<td>COS</td>
</tr>
<tr>
<td>Consensus Assessment of Sequence</td>
<td>CASAVA</td>
</tr>
<tr>
<td>and Variation</td>
<td></td>
</tr>
<tr>
<td>Cryo-electron tomography</td>
<td>Cryo-ET</td>
</tr>
<tr>
<td>Cyclic guanosine monophosphate</td>
<td>cGMP</td>
</tr>
<tr>
<td>Deoxyribonucleotide triphosphate</td>
<td>dNTP</td>
</tr>
<tr>
<td>Dihydriodipicolinate reductase</td>
<td>dapB</td>
</tr>
<tr>
<td>Dihydroethidium</td>
<td>DHE</td>
</tr>
<tr>
<td>Distal invaginations</td>
<td>DI</td>
</tr>
<tr>
<td>Dulbecco’s modified Eagle’s</td>
<td>DMEM</td>
</tr>
<tr>
<td>medium</td>
<td></td>
</tr>
<tr>
<td>Efficient Large-Scale Alignment</td>
<td>ELAND</td>
</tr>
<tr>
<td>of Nucleotide Data-bases</td>
<td></td>
</tr>
<tr>
<td>Enhanced S-cone syndrome</td>
<td>ESCS</td>
</tr>
<tr>
<td>Electroretinography</td>
<td>ERG</td>
</tr>
</tbody>
</table>
Fluorescein isothiocyanate-FITC
Focused ion beam-FIB
Fragments per kilobase of exon model per million mapped reads-FPKM
G-protein coupled receptor-GPCR
Ganglion cell layer-GCL
Glutathione peroxidase-GPX
Glutathione S-transferase-GST
Green fluorescent protein-GFP
Gene ontology-GO
Heat shock protein-HSP
Immunohistochemistry-IHC
Inner nuclear layer-INL
Inner plexiform layer-IPL
Interferon-IFN
Intraperitoneal-IP
Inner segment-IS
L-cone-long wavelength sensitive cone photoreceptor
Long intergenic non-coding RNA-LineRNA
M-cone-medium wavelength sensitive cone photoreceptor
Metallothionein-MT
Monoclonal antibody-mAb
Mouse cone ultraviolet-MUV
N-retinylidene-N-retinylethanolamine-A2E
Neural retina leucine zipper-Nrl
Outer nuclear layer-ONL
Outer segment-OS
Peanut agglutinin-PNA
Phosphate buffered saline-PBS
Phosphodiesterase-PDE
Phosphatidylserine-PS
photoreceptor layer-PR
Photoreceptor outer segments-POS
Platinum-Pt
Polyclonal antibody-pAb
Quantitative RT–PCR-qRT-PCR
Quantitative trait loci-QTL
Quinolinium,4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-, diiodide-ToPro3
Reactive oxygen species-ROS
Real time–polymerase chain reaction-RT-PCR
RNA-Seq-RNA- Sequencing
Rod outer segment-ROS
RPE-retinal pigmented epithelium
S-cone-short wavelength sensitive cone photoreceptor
Scanning electron microscopy-SEM
Scanning laser ophthalmoscopy-SLO
Semi–quantitative RT–PCR-sqRT-PCR
Serial block face-SBF
Single nucleotide polymorphism-SNP
Spectral domain-optical coherence tomography-SD-OCT

xviii
Thyroid hormone receptor β2-TR-β2
Transmission electron microscopy-TEM
Two-photon microscopy-TPM
Ubiquitin c-Ubc
Wheat germ agglutinin-WGA
Wild-type-Wt
Genetic Signatures of the Retina in Health and Disease

ABSTRACT

by

DEBARSHI MUSTAFI

The first steps in vision begin with signaling by photoreceptor cells of the retina. These post-mitotic neurons are reliant on the neighboring retinal pigmented epithelium (RPE) for daily homeostatic functions for life-long maintenance. Pathology affecting these integral cell layers can give rise to various retinal pathologies, with varying genetic patterns ranging from rare Mendelian diseases to more common complex multi-genic diseases. The goal of this work was to understand the phenotypic and genotypic factors that contribute to normal physiological function of the retina and the changes that manifest in disease. Human patients and mouse models of the Mendelian disease enhanced S-cone syndrome were subjected to RNA-Sequencing (RNA-Seq) and high-resolution hybrid microscopy methods to understand the genomic features that result in the progressive degenerative phenotype. It was revealed that a defect in proper photoreceptor shedding and phagocytosis, stemming for the aberrant development of these photoreceptors, gave rise to the progressive degeneration. Expanding the work to a more common, yet complex retinal degeneration, age-related retinal degeneration (ARD), the importance of genetic epistasis in disease was revealed. The use of inbred mouse lines with differing genetic backgrounds revealed that subtle differences in levels of inflammatory and RPE homeostatic factors can have profound effects on the predisposition and pathogenesis of ARD. The RNA-Seq from these studies revealed that non-coding RNAs may also mediate phenotypic diversity in disease. An evolutionary analysis of long intergenic non-coding RNA (lincRNA) across species with varying retinal environments elucidated those lincRNAs that were conserved in sequence. Further biochemical and bioinformatic experiments revealed that these lincRNAs may be critical for retinal physiology in adults and may represent another level of transcriptional regulation in the genetic architecture of the eye in health and disease.
CHAPTER 1: THE GENOTYPE TO PHENOTYPE QUESTION UNDERLYING VISION

Portions of this chapter were previously published in:


1.1 The eye

1.1.1 Features of the eye across species

Vertebrate eyes are all based on a common structural plan. The visual pigment genes are all descended from the same remote ancestor despite differences in embryological development and optical layouts among invertebrates. Furthermore, genes involved in formation of the vertebrate eye have proved to be homologous with those of the *Drosophila* eye (1), strongly suggesting that, despite certain differences, eyes across vertebrates have a common origin, with *Pax6* as potentially the universal master control gene for eye morphogenesis (2, 3). Differences among vertebrates, therefore, must reflect adaptation to particular environments and appropriately visual capabilities of animals have evolved to match aspects of their photic environment. At the most basic level, nocturnal animals have the most rod-dominated retinas, whereas diurnal species have more cone-rich retinas. One of the most striking modifications of the ancestral pattern of four spectral classes of cone opsins is found in mammals, where only the two spectrally extreme classes are present. One explanation for this intermediate class loss relates to the evolution of mammals when reptilian ancestors went through a prolonged nocturnal phase. It is thought that because genes have no long-term storage mechanism, a gene cannot be retained unless it continuously remains functional, although there are exceptions to this idea, as supported by the blind cavefish (4). Among mammals, only primates have evolved trichromatic color vision. The primary mechanism for trichromacy in New World primates is through allelic diversity of the L/M cone opsin gene on the X-chromosome. This single visual pigment gene has multiple alleles. Heterozygous females segregate expression of the alleles into separate populations of cones that are
trichromatic. Old World primates, including humans, have evolved trichromatic vision through gene duplication and divergence of the cone opsin gene on the X-chromosome. Primates are trichromatic with three cone pigments (5) termed L, M, and S, distinguished mainly by the portion of the visible spectrum to which each is maximally sensitive. L cones are most sensitive to low-frequency photons ($\lambda_{\text{max}} \sim 555-565$ nm), M cones to middle-frequency photons ($\lambda_{\text{max}} \sim 530-537$ nm), and S cones to supra-frequency photons ($\lambda_{\text{max}} \sim 415-430$ nm). Compared to L and M cones, inner segments (IS) of S cones are slightly extended. On average, there are over twice as many L-cones than M-cones in humans, but Old World primates do exist that exhibit more variation with some actually exhibiting M-cones that outnumber L-cones (6); otherwise these two cone types show similar spatial distributions and appear to be randomly intermixed. L and M cones are most concentrated in the fovea where they are densely packed in a hexagonal pattern that accounts for the high visual acuity capability of the fovea. However, the spatial distribution of S cones across the retina differs from that of L and M cones in several respects. S cones constitute only about 5% of the total cone population (7), they are more peripherally located in the retina and are absent from the center of the human fovea. Indeed, pigments in the lens and macula selectively reduce the fraction of higher-frequency photons that reach the retina and, hence, the photoreceptors, thereby reducing the need for S-cones. This ‘filtration’ process improves vision in two respects. First, removal of higher-frequency photons serves to sharpen the image (due to the refractive properties of water in the interior of the eye). Second, such ‘filtration’ reduces damage to the retina and photoreceptors produced by high frequency photons. Because most high frequency photons are absorbed by the lens, images are produced mainly by L and M
cones with less of a contribution from S-cones, which are nonetheless important because they contribute to color in image formation.

1.1.2 The retinal layer and the role of rod and cone photoreceptors

In the posterior of the eye lies the retina, which is the tissue layer that converts light into visual signals transmitted to the brain. This process is carried out by two major types of photoreceptors, rods and cones that are distinguished by their shape, type of photopigment, retinal distribution, and pattern of synaptic connections (Figure 1). These properties reflect the fact that rod and cone systems are specialized for different aspects of vision. The rod system has low spatial resolution but is extremely sensitive to light so it is specialized for sensitivity at the expense of resolution. Conversely, the cone system has high spatial resolution but is relatively insensitive to light. Thus, it is specialized for visual acuity at the expense of sensitivity. The different architectures of their outer segments (OS) represent a major distinctive feature of these two cell types. Rods with their longer OS composed of individualized discs unconnected to the ciliary plasma membrane contrast starkly with cones, which features shorter OS that arise initially as evaginations with subsequent formation of a series of discs (or invaginations), which are continuously connected to the membrane of the cilium that extends over the length of the OS. Lack of rim formation is the reason for this open formation of cone discs (8).

The functional differences that underlie these photoreceptors extend beyond their distinct structural features. Arrangement of the circuits that transmit rod and cone information to retinal ganglion cells contributes to the different characteristics of scotopic (rod) and photopic (cone) vision. Pathways linking rods and cones to ganglion cells are
largely independent at early stages. A striking difference between rod and cone circuitry is the degree of their convergence. Each rod bipolar cell is contacted by a number of rods, and many rod bipolar cells contact a given amacrine cell. In contrast, the cone system is much less convergent. Thus, each retinal ganglion cell that dominates central vision receives input from only one cone bipolar cell, which in turn, is contacted by only a single cone. More convergence makes the rod system a better detector of light, as small signals from many rods are pooled to generate a large response in the bipolar cell. However, such convergence also reduces the spatial resolution of the rod system. The one-to-one relationship of cones to bipolar and ganglion cells is just what is required to maximize visual acuity. Furthermore, the ability of rods and cones to respond to various ranges of light intensity results from different transduction mechanisms utilized by these two receptors. For example, a rod produces a reliable response to a single photon of light, whereas more than 100 photons are required to produce a comparable response in a cone. This difference does not reflect failure by cones to capture photons effectively. Rather, the change in current produced by single photon capture in cones is comparatively small and difficult to distinguish from background noise. Another difference is that the response of an individual cone does not saturate at high levels of steady illumination, as does the rod response. Finally, compared to cones, rods show little, if any, directional sensitivity.

1.1.3 The importance of cone cells in the context of retinal disease

A host of genes and their protein products are necessary for the structural and functional make up of these photoreceptors. Disruption in these genes and proteins can result in loss of structural and functional integrity, manifesting as loss of vision.
Photoreceptor degeneration results in vision loss in diseases like retinitis pigmentosa and age-related macular degeneration. In these diseases, the main cause of clinically significant vision loss is cone cell degeneration rather than rod cell death. Although most mutations responsible for retinitis pigmentosa in humans and animal models affect rod-photoreceptor-specific genes, rod apoptosis is often followed by secondary cone degeneration (9). Nevertheless, people with a night blindness disease can lead a normal life, especially in industrialized countries, because they can still see satisfactorily despite the loss of rods. The secondary loss of cone photoreceptor brings more clinically significant symptoms in patients thus, prevention of cone cell loss is a major goal of therapeutic strategies (10). Therefore, a complete understanding of cone cells is required, at both the structural and functional level, to accurately design therapeutics to rescue their function in different retinopathies, whether they are congenital or acquired.

1.2 Cone phototransduction signaling

1.2.1 The first steps in visual response by cones

Of light incident on the eye, about 75% reaches the fovea, which is the region that triggers the greatest degree of visual acuity, responds to higher light intensities, and where there is the greatest cone photoreceptor density (Figure 2). The image that falls on the retina is sufficiently sharp that a single cone can encounter significantly more photons than an adjacent one. Of the photons reaching the fovea, about one-quarter of the photons fall within an inner region that contains about 30 cones (11). Cones in outer regions of the fovea capture the rest of the photons. Outside the fovea, the rest of the surrounding
eye is predominantly populated by rods. The probability that a photon will be absorbed by a cone is based on three factors in temporal order:

1. The direction of arrival of the photon (most efficient when along the long axis of cone).
2. The frequency of the photon (only ~67% are of the correct frequency to photoisomerize visual pigment molecules due to the spectral content of the signal).
3. The type of cone (L, M, or S-type).

Cone phototransduction is a complex process that has been elucidated mechanistically (12-17). Light modulates depolarization of cones by increasing the rate of glutamate neurotransmitter release whereas hyperpolarization decreases this rate. Light causes hyperpolarization whereas darkness causes depolarization, so that maximum release of the neurotransmitter occurs in the dark and the action of light is to reduce this rate of release. The only actions of a photoreceptor that directly affect horizontal and bipolar cells are the release of glutamate by the nerve terminal and the removal of this transmitter via reuptake mechanisms. Looking downstream, whereas a rod cell has a single synaptic ribbon and contacts no more than seven or so processes of horizontal or bipolar cells (Figure 1), each cone cell contains many synaptic ribbons and contacts hundreds of such processes. The in-coming photon must first initiate the phototransduction cascade to facilitate transmission of the light signal through these downstream cells.

The process of phototransduction can be broken down into several general steps (Figure 3). The first is photoactivation where 11-cis-retinal, the chromophore for both rod and cone photoreceptors, is photoisomerized to the all-trans-retinal isomer, thereby
inducing a conformational change in the structure of the opsin protein molecule (Figure 3B) (18, 19). This conformational change allows the catalytically active opsin to bind to transducin, a G-protein, to initiate phototransduction. Binding of opsin to transducin replaces the GDP with GTP activating the α-subunit of transducin which then dissociates to activate the membrane-associated phosphodiesterase (PDE) by removing the two regulatory (γ) subunits (Figure 3C). In the dark, the OS cation channels are gated by cyclic guanosine monophosphate (cGMP), controlling the influx of ions across the photoreceptor plasma membrane. The hydrolysis of cGMP by PDE results in channel closure, thus decreasing the conductance of the plasma membrane to cations, which hyperpolarizes the plasma membrane, inhibits neurotransmitter release, and signals the adjacent neurons of the light stimulus (20). The disc is an important structural component in this step, and in the cone, the disc shape and composition is a critical determinant of its extended activity in light. In cones, the protein molecules of the biochemical cascade are similar to those of rods, but they are located for the most part on open discs that are continuous with the plasma membrane, rather than on discrete disc membranes (Figure 4). This allows membrane proteins to diffuse among different cone discs. In the second step, there is a decreased release of the excitatory neurotransmitter, glutamate. In the third and final step, cone photoreceptors cells recover from the photoresponse through a series of quenching/termination reactions of all activated phototransduction proteins to bring these cells back to their dark-adapted state (Figure 3D).

1.2.2 Structural dynamics of cone opsins during phototransduction

Rods and cones have four primary structural/functional regions: OS, IS, cell bodies, and synaptic terminals. Similar to humans, murine rod discs are completely
internalized and therefore physically separated from the cellular plasma membrane, whereas cone discs are delineated by foldings of the plasma membrane itself (Figure 4). Thus, open cone discs offer a much larger surface area for rapid exchange of substances between the cell exterior and interior, such as chromophore transfer for pigment regeneration and fast calcium dynamics during light adaptation (21). Cone outer segments (COS) possess open discs, which are continuous with the plasma membrane of the connecting cilium whereas OS of rods are separated from the plasma membrane. This correlates with the observations that deletion of peripherin/rds, a protein present in both cone and rod OS required for normal OS disc morphogenesis (8, 22, 23), produced nonfunctional rod precursors that undergo apoptosis, whereas cones remain viable, despite the development of atypical OS with reduced phototransduction efficiency (24-26).

Opsins, as a subclass of G-protein coupled receptors (GPCRs) (19), are defined by their ability to bind a retinal-based chromophore in order to form a light-sensitive photopigment (27). Properties that differ among the various opsin classes suggest biochemical and structural differences among opsin classes (28). Hydroxylamine bleaching susceptibility of cone opsins as contrasted to rhodopsin in the dark state (29, 30) implies that cone opsin classes may have a relatively open conformation in the dark that allows hydroxylamine to compete with opsin for binding to 11-cis-retinal. Also, site-directed mutagenesis studies have shown that each opsin class may have different residues that affect its overall stability (31). Despite these differences, rhodopsin and transgenic rod/cone pigments employ identical downstream signaling mechanisms when compared side-by-side in Xenopus rods and cones (32). The same was reported for
rhodopsin and transgenic red/cone pigments in mouse rods (33). Thus, not only do rod and cone pigments interact with a given transducin identically, but the shutoff mediated by a given protein kinase and arrestin is also similar (21). Despite these observations the three-dimensional structure of COS needs to be elucidated by more advanced techniques such as cryo-electron tomography (cryo-ET) as has been done for ROS to reveal an accurate structural framework for the space within which phototransduction occurs (34).

Even as rhodopsin comprises ~90% of the protein in rod disc membranes, the composition and organization of opsins in cone cells have yet to be determined. Such information would be broadly applicable to other signal transduction cascades because GPCRs represent the largest known class of drug, hormone and neuropeptide receptors. Homology modeling of cone opsins with the X-ray defined structure of rhodopsin (28) reveals that the three cone opsins are similar. The S-cone opsin homology model is shown to illustrate the structural elements. Key amino acids in the retinal binding pocket shown for the blue cone opsin indicate that the central residue Tyr262, is much different than the central residue of Trp281 (analogous to Trp262 in bovine rhodopsin) in red and green cone opsins (Figure 5). Moreover, the binding pocket of the blue cone opsin lacks a glutamate residue to act as a counter ion to the chromophore Schiff base, resulting in the blue shift of this opsin.

1.3 Evolutionary dynamics of the eye
The steps of cone phototransduction provide insight into the specialized function of this neuron in vision, but to appreciate its role in the context of the entire retina, an understanding of retinal and ultimately cone cell evolution is required.

### 1.3.1 Evolution of retinal circuitry

Early photoreceptor cells that evolved into the current rod and cone structures were the ciliary and rhadomeric cells. The evolutionary relevance of rhadomeric photoreceptors to rods and cones has recently been elucidated in invertebrates (35) and clarified in vertebrates by the proposal that vertebrate retinal ganglion cells actually are daughter cells of rhadomeric photoreceptors, found mainly in the compound eyes of arthropods. Unlike ciliary cells where modification of the cilium increases the membrane surface area, rhadomeric cells lacking cilia increase their surface area through microvilli. This view is based primarily on the close homology of transcription factors used by the two classes of cells. Secondly, melanopsin, an opsin integral to circadian control found in special retinal ganglion cells that are depolarized rather than hyperpolarized by light, is a member of the rhadomeric class of opsins (36-38). Therefore, a reasonable assumption is that modern mammalian retinal ganglion cells actually are rhadomeric photoreceptors that have lost their original membrane structure, but have retained their axons, their ancestral responses to neurogenic factors and, in some cases, their rhadomeric opsin and G protein-signaling cascade. Modern ganglion cells also have acquired the ability to receive synaptic input from ciliary photoreceptors, which have evolved into present day cone and rod cells. Additionally, evolution has provided a gradual transition towards a highly organized laminar structure of the OS and the appearance of ribbons in the synaptic terminals (39-43). Much about the development of the circuitry in the
mammalian retina is known from studying ferrets, which are convenient to work with because their young are born at a very immature developmental stage with eyes that do not open until 2 weeks after birth (44) (Figure 6).

1.3.2 Evolution of cone photoreceptors

Both autoradiographic and kinetic data of COS renewal (45) predict that many opsin molecules in COS will become much older than the oldest opsin molecules in ROS. The presence of a significant number of older molecules within COS membranes is not detrimental to cone function, because cones function in daylight and signal the absorption of thousands to millions of photons despite a high level of noise (46). But even so, the efficiency of COS renewal mechanisms may limit the sensitivity of a photoreceptor to light (45). Although the actual turnover rate of cones compared to rods is unknown, it is thought that rods exhibit more rapid decay than cones. The slow turnover of COS actually is typical of renewal rates for most intracellular components in a majority of cells. The highly efficient OS renewal mechanism in rods is thus unusual, suggesting a specialized evolution from cones, rather than the opposite. This fundamental difference between ROS and COS strongly suggesting that rods are modified cones is supported by the following observations:

1. Most vertebrates develop COS prior to ROS and the initial topology of developing photoreceptors is cone-like (47, 48).

2. Vertebrate retinas have many cone types, but fewer rod types (49).
3. Close amino acid homology between photoreceptor opsins indicates that genes encoding the three human cone pigments and rhodopsin are all derived from a common ancestral gene (50).

These past insights into cone evolution have been substantiated by studies of the transcriptional network that regulates photoreceptor development. One of these transcription factors central to our understanding of cone cell development, is named the neural retina leucine zipper (Nrl). The Nrl gene was identified by subtraction cloning and detected only in the neural retina, including the photoreceptor cells and inner nuclear layers (51) with Nrl knockout mice being functionally ‘rodless’ with photoreceptors that adopted cone-like properties (52-56).

By using the Nrl-promoter to express green fluorescent protein (GFP) in transgenic mice, researchers have shown that Nrl is indeed one of the earliest rod lineage-specific markers. To evaluate the origin of enhanced S-cones in the Nrl+/− retina directly, wild type-GFP mice were crossed with Nrl−/− mice. GFP+ cells (rod precursors in wild type retina) were also co-labeled with S-opsin in Nrl−/− GFP retinas. Because Nrl−/− mice produce only S-cones, it was proposed that Nrl determines the rod fate of “bipotent” photoreceptor precursors by modulating gene networks that simultaneously activate rod- and suppress cone-specific genes. In the cone development pathway, a second transcription factor, thyroid hormone receptor β2 (TR-β2) regulates the developmental “choice” between S-opsin and M/L-opsin expression; mice without a functional TR-β2 have no M-opsin expressing cones (57, 58). A percentage of early cones are thus directed to an M-opsin expressing fate by TR-β2; unliganded TR-β2 acts to repress S-opsin
expression in cones, whereas liganded TR-β2 activates M-opsin expression (57), with a contribution from the retinoid X receptor (59).

More proof for this hypothesis is provided by retinal disease profiles. Immunohistochemical and physiological studies (60) suggest that Nrl modulates the development of S-cones, and that its gain or loss of function primarily results in alterations of the S-cone pathway. One possible explanation is that S-cones represent the “default fate” for early-born photoreceptors in mice (61) and that the expression of Nrl controls an important node in this process. These results are consistent with evolutionary data suggesting that rods are derived from an ancestral cone (62). There is even further evidence from the branching pattern of vertebrate retinal opsins that classes of cone pigments existed before evolution of the rod pigment, rhodopsin (30). The order in which different classes of retinal cells are generated is generally conserved across vertebrate species. During the first wave of cell genesis, retinal ganglion cells, horizontal cells, amacrine cells, and cone cells are born. A subsequent wave produces the majority of rods, the remaining amacrine cells, the bipolar cells and the Müller cells as well (63).

1.4 Structural features of cone photoreceptors

The evolutionary perspective of cone photoreceptor function has been further corroborated by structural investigations. It has been demonstrated that the retinal pigmented epithelium (RPE) extends long processes that reach cone tips. Tubular processes protrude from the apical surface of the RPE to ensheath the COS (64). This early description was later confirmed by EM data (65) and subsequently by ultrastructural
studies of this relationship in human extrafoveal cones (66). Because ROS are cylindrically shaped and each rod disc becomes independent after its initial formation at the OS base, continuous displacement of discs toward the OS tip is easy to visualize in three dimensions. If one presumes that cone discs are also displaced, the process must be more complex because many, and perhaps all, discs retain some connection with not only the adjacent discs, but also the outer plasma membrane (67, 68) (Figure 4).

1.4.1 Cone disc renewal

The availability of rod dominated retinal samples from mouse models has allowed us to understand not only rod cell structure, but also the steps inherent for renewal of its discs. ROS are renewed in an orderly fashion, as first revealed by autoradiographic studies in which radioactive protein molecules became trapped in new membranous discs generated at the OS base, producing autoradiographically labeled bands. Unchanged bands were displaced sclerally as additional discs formed below and finally were discarded from the OS tip and phagocytized by the RPE (69). This was further supported for the first time in 1969 with biochemical work (70). These and other studies (71-73), indicate that new membrane is incorporated into ROS via the connecting cilium by distributing into successive new membrane folds that evaginate from the cilium at the OS base. These evaginations expand to the full OS width and are displaced away from the base. They then lose their connections and become isolated into separate discs, all surrounded by the plasma membrane. An alternative mechanism was recently proposed in which vesicles fuse to form nascent discs that are assembled entirely within the cell’s membrane (74). This fusion model, however, assumes that nascent discs are closed and
do not differ from the mature discs except in size. This membrane fusion idea possibly stemmed from a preservation-artifact as previously described (75).

COS differ from ROS in structural organization, autoradiographic labeling pattern, and three-dimensional shape. COS consist of numerous parallel membrane foldings oriented at right angles to the connecting cilium that apparently retain continuity with each other and with the plasma membrane, forming a single topologically continuous membrane system in some species. Early studies revealed the tapered conical shape of COS and presented circumstantial evidence that their membranes are not renewed due to their mode of development (76). Since then, it has been generally accepted that the tips of mature COS are regularly shed, and therefore their membranes must be shed as well. Presumably, membranes in COS are renewed in a manner unlike ROS due to these structural differences, but it is not completely understood. Although cones are more difficult to study than rods, it is especially important to clarify how COS are renewed because humans rely much more on cone-based day vision than on rod-based night vision. Instead of distinct discs, COS in all vertebrate retinas share the structural feature of numerous parallel lamellae connected by a single longitudinal ciliary stalk (67, 72, 77). Most lamellae in a COS extend laterally across the full COS width, but a few at the base are incomplete (Figure 4).

In non-mammalian cones, the OS are composed of a stack of discs, all of which are continuous with each other and with the outer membrane adjacent to the connecting cilium (78) (Figure 4). In mammalian cones, however, only the basal part of the OS seems to retain this organization in single thin sections (67). It was initially thought that displacement of cone discs from the base to the OS tip must be accompanied by a similar
displacement of the cilium and outer membrane, because otherwise connections between the discs and outer membrane would have to continually be formed and broken to permit disc displacement. Coordinated displacement, in turn, indicates that the outer membrane must also be continually replaced in conjunction with the discs. That this actually might occur for both rods and cones steamed from evidence that shed disc packets from both photoreceptor types are surrounded by shed outer plasma membrane (66, 79). This understanding of disc displacement in cones has been revised with more current work that embraces the idea that because the autoradiographic data suggested that protein is randomly distributed, protein and lipid components must freely flow throughout the system and that there is terminal loss of discs from the OS and phagocytosis by RPE cells as demonstrated by ground squirrel, monkey, and human cone turnover (67).

The entire COS appears to be continually remodeled or reshaped as a unit, from the time of its initial outgrowth until its mature dimensions and shape are achieved, a process supported by the open disc structure of COS. It is thought that mature COS remain tapered despite shedding because their distal lamellae shrink by losing membrane (73). The entire COS is thought to expand in all three dimensions during morphogenesis. The COS shape changes because the length increases more than the width. Rather than requiring shrinkage of lamellae at the tip, the change in taper of developing COS can be interpreted as reflecting differential growth (80). Actin is thought to be involved in the formation of evaginations at the base of the OS and this has been substantiated by examining how the microfilament-destabilizing drug, cytochalasin D, affects photoreceptor OS morphology. No new discs/lamellae seem to form at the OS base in the presence of this inhibitor (81, 82). However, these findings must be interpreted with
caution because the basic mechanism is unknown and the main drug effect may be disruption of actin filaments at the COS base that indirectly interferes with processes that occur more distally.

1.4.2 Cone disc morphogenesis

Disc morphogenesis in ROS is quite different than in COS, giving rise to the more open cone discs that permit continuous protein flow. ROS lamellae are formed successively and discs become isolated from the plasma membrane near the ROS base in young rods. In contrast, the first membrane foldings during OS development in cones arise as evaginations of the ciliary membrane because the cilium is the only structure initially present (73, 83). The prevailing theory of cone morphogenesis has evolved over time leading to the present day theory that cone disc structure is related to incomplete rim development.

Earlier it was thought that as cones develop, lamellae can expand simultaneously at many levels of the COS (84). In 1987, studies of *Xenopus* COS revealed unique structures termed distal invaginations (DI). In these early EM studies, it was shown that some of the distal folds of the disc margins are incomplete in that they extend from the non-ciliary side of the COS only part way across its width (Figure 4). These structures were termed DI because they are invaginations of the plasma membrane that occur throughout all basal COS levels. The presence of DI causes minimal interruption of the regular spacing between COS lamellae, but complete lamellae above and below a large group of DI are oblique, rather than parallel to one other (85). DI were not observed within the distal membranes of developing ROS. This was consistent with previous
research indicating that all new membranes in the developing ROS are assembled in the basal evagination (BE) at the ROS base, so that additional membrane cannot flow into ROS discs that have been separated from the plasma membrane (73).

The variable amount of taper of different COS can result from variation in the relative amounts of new membrane that flow into the BE versus the DI. This idea was based on COS that are highly tapered (as in amphibian retinas) such that they form few BE and many DI. However, COS can be slightly tapered (as in mammalian retinas) if they form many BE and few DI and in some cases OS can be cylindrical (as are ROS) if they form only BE (86). Such variable degrees of taper indicate that the idea of DI may not be a correct unifying theory for cone disc formation and it was actually hinted to be a possible mechanism for cone disc resorption and recycling (87). More recent work on the localization of peripherin/rds, specifically in the disc rim region of cone disc membranes (8, 25, 26), explains previous data from mammalian cones where only the basal part of the cone OS seems to retain this organization in single thin sections (67). This also indicates that the more gradual development of the disc rim in cones (73) gives rise to the open structure, permitting the nascent disc zone to extend further distally. Studies of peripherin/rds are consistent with the new proposed theory that the rim development is a slow, incomplete process arising from the cilium.

The cilium is clearly important for normal photoreceptor function, especially for cones, because the OS develops as an elaboration of this structure and cone discs are more intimately connected to it than rod discs. The cilium is the major cytoskeletal element of the OS in mature photoreceptors and also is the only cytoplasmic connection between the IS and OS. Thus, the cilia constitute the major route through which
materials, such as opsins (88), synthesized in the IS are delivered to the OS. So it is hardly surprising that human diseases with ciliary defects may result in retinal degeneration and night blindness (89-93). The importance of OS assembly and maintenance in its function is supported by the findings of intraflagellar transport proteins (94) and the detrimental effects seen when these protein complexes are mutated (95, 96). More importance of the cilium to cones may be explained by the fact that the relative length of the ciliary axoneme is different in ROS than COS (Figure 4). In COS, the axoneme is thought to extend the entire length of the OS (97), whereas in the ROS, the axoneme extends for most, but not the entire length (98). However, there is evidence that, aside from the axoneme in the ROS, there are distinct microtubule-like structures distally that extend the over the remaining length of the OS. These structures seem to be modulated by light-induced interactions between the RPE and photoreceptors, as occurs with disc shedding (99). Despite the great strides made in understanding the cone cell function and structure, shortcomings arise from the lack of appropriate animal models to study cone cells.

1.5 Mammalian models to better elucidate cone cell structure and function

Lack of a suitable experimental mammalian model constitutes the major impediment to understanding cone pathophysiology. Nocturnal species like laboratory rats and mice, which are useful for a variety of studies, have little place in cone photoreceptor research due to the dearth of cone cells in these species (100, 101). However, animals with cone dominance such as ground squirrels (~85% cones) (102-104), chickens (~65% cones) (105), and pigs (~20% cones) (106) not only are hard to breed in captivity, but also
cannot be studied with pre-existing antibodies generated for rodent cone targets. Therefore, despite the cone-dominated retina in these species, they do not serve well for scientific research purposes. A cone-rich transgenic mouse model and a similar cone-rich natural diurnal rat should provide scientists the ability to work with cone dominant animals that are not only easy to breed, but also will allow preexisting well developed technologies to be exploited to their fullest extent.

1.5.1 Mouse models to study cone cells

In addition to elucidating cone cell evolution, the Nrl<sup>−/−</sup> mouse model provides an ideal opportunity to study cone cell structure and function. The photoreceptors from these mice resemble cones (107) and possess cone-like characteristics (108). However, it is clear that these cells are hybrid between rods and cones. Key molecules of the cone phototransduction cascade, i.e., mouse cone ultraviolet (MUV) pigment, cone transducin, and cone arrestin—are present at cellular concentrations comparable to those of homologous transduction proteins measured in rods and expected to be present in wild type cones. Also, proteins of the cone phototransduction cascade in Nrl<sup>−/−</sup> photoreceptors drive photoresponses with high efficiency and cone-like recovery kinetics. Nonetheless, Nrl<sup>−/−</sup> photoreceptors are not a perfect model of normal wild type mouse cones as proved by apparently disordered and deteriorating cones with OS shorter than wild type cones that express “rod” arrestin. Photoreceptor function is healthy and stable in the Nrl<sup>−/−</sup> retina during the 4 to 6 week period after birth but it deteriorates subsequently, as displayed by a decline in the maximal amplitude of the a-wave (108), which is the initial negative deflection in response to a bright flash. Despite this decline, each Nrl<sup>−/−</sup> photoreceptor, like wild type mouse cones, has an associated peanut agglutinin (PNA)-stainable sheath.
This observation supports the intriguing hypothesis that the sheath is secreted by cones themselves because Nrl\(^{-/-}\) photoreceptors are far removed from the RPE cell apical surface. These limitations should not detract from the contribution of this species to the understanding cone ultrastructure. Detailed study of the Nrl\(^{-/-}\) mouse model can also lead to a better understanding of diseases that result in over production of cones such as enhanced S-cone syndrome (ESCS), which is a unique retinal disease. Loss of visual function in hereditary human retinal degenerative diseases usually reduces photoreceptor cells by apoptosis (109), but the one exception is ESCS, manifested as a gain in function in photoreceptor development (110-114).

### 1.5.2 Other mammalian rodent species for study of cone cells

Another promising species is the Nile rat (Arvicanthis niloticus). This newly studied rodent has a diurnal behavior pattern similar to humans and it also uniquely possesses a large percentage of easily identifiable cones (~33%) (115, 116). At the ultrastructural level, the RPE-OS interface exhibits an orderly arrangement of ROS and COS apposed to the RPE apical surface. The ROS appear as cylindrical structures with clearly visible stacked discs. The COS are narrower and tapered, with areas of clear cytoplasm and a surrounding cone matrix sheath (115). More importantly, the cones can be conveniently stained with antibodies raised against murine peptide sequences and other proteins involved in phototransduction, namely arrestin, recoverin and cGMP-gated channels (117). Studies with the Nile rat should build on research with Nrl\(^{-/-}\) species to establish improved cone photoreceptor structure-function relationships in mammalian species and further advance our understanding of the pathogenic changes that occur in cone cells in the context of disease.
1.6 The genotypic to phenotype connection in the retina in the context of disease

1.6.1 High resolution phenotypic methods to reveal retinal architecture

The mammalian retina consists of diverse cell types that have specific roles in visual processing. Techniques relying on light microscopy (118) in most cases cannot resolve neural network components that have diameters that are substantially below the wavelength of light (119). Newer super-resolution light microscopy (120) has overcome these depth resolution issues, but is strongly dependent on the property of the fluorophore used. Electron microscopy provides better resolution at much lower numerical apertures than light microscopy because of the small wavelength of electrons. The low numerical apertures, however, limits optical sectioning, therefore sections must be physically cut to view with electron microscopes. The use of electron microscopy techniques have been used in the retina to establish cell population counts (121) to assess alterations in retinal architecture in the face of congenital or acquired pathological states. These cell counts are based on fixed and stained retinas in plastic embedding that were serially sectioned by hand. This can result in distortions in the z-direction, limiting the reliability of the resulting reconstructions. Coupling automation of microscopy and sectioning offers the promise of reliably reconstructing neuronal tissues in the retina to accurately assess tissue architecture and the changes that result in disease.

Hybrid scanning electron microscopy (SEM) techniques coupled with focused ion beam (FIB) (122) or serial block face (SBF) (123) technology alleviates the distortion that accompanies transmission electron microscopy (TEM) serial sectioning because each tissue slice is imaged before sectioning. These two methods are very complementary as
FIB-SEM can image voxels of 4 x 4 x 10 nanometers compared to the 20 x 20 x 25 nanometer resolution limit of SBF-SEM, but the SBF-SEM can image much greater volumes, on the order of 2-3 orders of magnitude. Moreover, critical point-dried samples can be imaged using the FIB to visualize surface topography and internal architecture of retinal tissue (Figure 7A). The dual beam FIB system is a scanning microscope with an electron beam column and an ion beam column mounted on the same specimen chamber. The advantage of this dual beam technology is that the focused ion beam can be used for milling whereas the electron beam is used for SEM imaging (Figure 7B). The ion source (gallium) removes material from the surface of the specimen uniformly so it can be repeatedly imaged by the scanning electron beam. As a result, a trench is created, thus enabling imaging of the specimen’s interior (124-127) (Figure 8). In SBF-SEM, plastic embedded blocks are used. This methodology utilizes cutting of serial sections with an ultramicrotome inside the SEM chamber (123, 128) (Figure 9).

The detail afforded by these hybrid SEM techniques can be utilized to study the retinal architecture to deduce phenotypic effects. However accurate gene expression protocols will be necessary to assign genotypic contributions to perturbations seen in the retina at the structural level.

1.6.2 High throughput sequencing to reveal global gene expression patterns in the retina

Global gene expression profiles of the retina have been based on various techniques such as serial analysis of gene expression (129), expressed sequence tags (130), and hybridization microarrays (131), but the advent of RNA-Seq (RNA-Seq) (132) has revolutionized transcriptome analysis. Compared to previous methodology
in which only portions of a full transcript are analyzed and transcript isoforms are indistinguishable, RNA-Seq offers an unprecedented global view of the transcriptome (Figure 10). The single base resolution of RNA-Seq allows a more complete annotation of genes by revising the known exonic boundaries and intron sequences. Moreover, RNA-Seq is quantitative. It can be determine the absolute quantity of every molecule in a cell population (133) and directly allow comparison between experiments. This absolute quantification allows one to capture transcriptome dynamics (134) across different tissues or physiological changes that may accompany disease. RNA-Seq technology has also revealed a new level of complexity in the genome with the discovery of thousands of long non-coding RNAs (135), for which the functional roles remain undefined.

The use of this technology has already been now used to understand the mechanisms underlying human gene expression variation (136). Global gene expression profiles in the retina can revolutionize our understanding of diseases, both congenital and acquired, by identifying pathways and processes that are causally implicated in disease, and thus provide the first step in the discovery of target therapies. Moreover, improved genetic information regarding disease can lead to better design of prevention trials in the future (137).

**1.7 Project approach**

There is thus a pressing need to understand cone cells, especially in the context of retinal disease given the devastating clinical phenotypes associated with loss of cone mediated vision. Even in diseases characterized by primary rod photoreceptor loss, the secondary loss of cone cells are often more devastating to the patient as they lose high-
resolution central vision. Approaches to preventing these diseases begin by understanding the basic features of these photoreceptors. Studying mouse models of different retinopathies affecting cones can have direct correlations to human disease. This correlation can more thoroughly investigated by analyzing human patients with the disease and directly comparing it to a mouse model that approximates not only the genotypic features, but also the phenotypic features of the disease. Since mouse models can be studied more carefully and followed more closely, the connections between genes and phenotypes are therefore both more complete and systematic in model organisms than they are in humans, providing an unbiased assessment of the genetic complexity of phenotypic traits (138). By studying the in vivo state of the disease, the genetics of gene expression in the particular tissue or cell type in the tissue can reveal the complex interplay among many genomic loci (139). In depth RNA-Seq of mouse tissue can thus reveal variation and its effect on the phenotype (140). The phenotype of disease can be accurately followed up not only using light microscopy techniques, but higher resolution hybrid SEM techniques to highlight even subtle pathological changes that may be driving retinal disease. By interfacing high resolution imaging and high throughput genetics, this thesis project aims to understand features of congenital and acquired forms of retinal disease. Moreover, in light of the depth of sequencing obtained by RNA-Seq technology a goal of this project will be to investigate if outside of well annotated protein-coding transcripts, are there long non-coding RNA transcripts that may be implicated in adult retinal function and maintenance.
Figure 1. Differences in photoreceptors and their arrangement in the retina. Rod and cone photoreceptors are displayed in a cross-sectional depiction of the retina also showing connections of these photoreceptors to retinal pigment epithelium distally and relaying cells (bipolar, horizontal, amacrine, ganglion) proximally. Electron microscopic images are shown of a ROS (A) and a COS (B). The rod structure has a longer outer segment with discs packed without connections to the ciliary membrane, in stark contrast to the COS discs that are continuously connected by the ciliary membrane.
Figure 2. Distribution of photoreceptors in the eye. Overall, rods outnumber cones by a ratio of 20:1 or greater in the retina. However, in the fovea, the cone density is the highest and is correlated with visual acuity.
**Figure 3.** The steps in cone phototransduction. (A) In the dark state the cyclic nucleotide gated channels are open, but (B) upon light activation of opsin (R) to the activated stated (R*) causes a conformational change (C) to bind transducin and replace the GDP with GTP activating the $\alpha$-subunit of transducin which then dissociates to activate the membrane-associated phosphodiesterase (PDE). The hydrolysis of cGMP by PDE results in channel closure, thus decreasing the conductance of the plasma membrane to cations, which hyperpolarizes the plasma membrane, inhibits neurotransmitter release, and signals the adjacent neurons of the light stimulus. (D) Finally, cone photoreceptors cells recover from the photoresponse through a series of quenching/termination reactions of all activated phototransduction proteins to bring these cells back to their dark-adapted state.
Figure 4. Structure and renewal of rod and cone discs. Discs in the cone photoreceptor (A) are not unique evaginations completely separated from the cilium membrane, but instead retain connection to the cilium that extends the entire length of the outer segment. Early EM data indicated that discs of the COS feature partial folds composed of BE and DI. The arrow indicating growth away from the ciliary stalk represents a BE showing a developing COS surrounded by new membrane whereas arrows pointing toward the ciliary stalk represent more DI forming new but shorter COS. More recent work has shown that this representation is not a unifying model and the disc morphogenesis is more dependent on incomplete rim protein (peripherin/rds) formation. Regardless of the mechanism, the continuous membrane structure of cone discs permits an increased surface area that could explain a phototransduction cascade distinct from rod cells. (B) The cone axoneme (shown in red) extends the length of the cone ciliary stalk, indicating its importance in disc morphogenesis and turnover. The rod photoreceptor (C) features individualized discs that do not maintain any connection to the rod cilium. Furthermore, the ciliary axoneme (shown in red) does not extend the entire length of the ROS.
Figure 5. Homology model of S-cone (blue) opsin. (A) The S-cone opsin (pdb id: 1kpn) is shown in blue with the retinal chromophore shown as red sticks and Tyr262 side chain colored by atom. The shown opsin model is a homology based structure from bovine rhodopsin generated with the program Modeleer in the Insight II package. The only obvious differences between the two lie in the N- and C-terminal regions, with differences between the blue opsin and rhodopsin being minimal. Homology modeling of the other cone pigment opsins also revealed very similar differences. (B) The modeled retinal binding site is shown with the retinal depicted as red sticks and blue opsin residues as sticks colored by atom. The major difference seen between the three pigments is that, unlike the red and green opsins where the central residue forming the retinal cavity is Trp281 (analogous to Trp265 in bovine rhodopsin), the central residue forming the cavity in blue cone pigment is Tyr262 and there is no Glu residue to act as a counter ion to the chromophore Schiff base, resulting in the blue shift of this opsin.
Figure 6. Evolution of photoreceptors. This retinal model is based on the ferret, a mammal with a developmentally immature retina at birth. (A) Cones and rods have indistinct morphologies at birth with both photoreceptors extending processes to the inner plexiform layer (IPL). (B) At 2 weeks of age, morphologies are still indistinct but their processes retract and contact horizontal and bipolar cells. (C) Finally at 4 weeks or age, cones and rods are morphologically distinct with discernible inner and outer segments.
Figure 7. Schematics of retinal sample preparation and FIB-SEM based experimentation. (A) Enucleated mouse eyes were dissected and the retina carefully removed and separated from the retinal pigmented epithelium. The retina was then chemically fixed and incubated with heavy metal solutions consisting of osmium and uranium. Samples were dehydrated, critical point dried and placed on SEM stubs, the edges covered with conducting silver paste, and finally sputter coated with a 5-10 nm layer of gold to remove any charge build up at the surface. (B) Samples then were placed inside the FIB-SEM and tilted to 52° to accommodate accurate ion ablation (the ion column is positioned at 52° to the electron column). The principle is to image the surface with the electron beam, ablate off a thin layer (35-50 nm) with the ion beam, and then reimage the new surface with the electron beam.
Figure 8. Experimental set-up for photoreceptor observation with FIB-SEM technology. (A) Once an area of interest in critical point dried sample of *wt* mouse retina was selected it was protected by application of a thin layer of platinum inside the FIB-SEM (indicated by arrow), a trench was created by application of an ion beam cleaning cross section. (B) A close-up view of the created trench reveals interior elements of rod photoreceptors that can be imaged by the electron beam. Scale bars in panels A and B are 50 and 5 μm, respectively.
Figure 9. Schematic of SBF-SEM experimentation. Plastic embedded mouse eye cups are placed inside the SEM. The surface was imaged using and then the microtome inside the chamber cut a section (70-100 nm in depth) and the resulting face was imaged again by the SEM. This allowed collection of serial sections of the block face for downstream analysis and three-dimensional reconstructions of the retina.
Figure 10. High-throughput sequencing reveals global gene expression patterns. RNA-Seq of mouse eye tissue shown reveals coverage across the entire transcriptome. Moreover, the depth of sequencing allowed one to not only look at individual chromosomes, but also at the reads encompassing single genes and quantitate the gene expression levels.
CHAPTER 2: PROGRESSIVE DEGENERATION IN MONOGENIC ENHANCED S-CONE SYNDROME IS DRIVEN BY ABBERRANT RETINAL HOMEOSTASIS

Portions of this chapter were previously published in:


2.1 The monogenic disease enhanced S-cone syndrome

2.1.1 The human condition and the corresponding mouse model of disease

The retina of all vertebrates contains two sets of photoreceptors that are unique to the lighting conditions that are encountered. Rod photoreceptors serve for dim light environments and cone photoreceptors for brighter environments; cones are further divided into short-wavelength sensitive (S-) and long- and middle-wavelength sensitive (L/M-) sub-types. These photoreceptors, which are organized into mosaic structures with characteristic rod/cone ratio in retinal position and species specific manner, are organized to provide useful visual sensation for the organism during their entire life span. Thus, a normal retina is controlled by a multitude of interacting factors that determine the precise developmental organization and lifetime maintenance of interconnected neurons for optimal visual function (141, 142). Disruption of these complex interactions during development or in the mature retina can give rise to cellular pathology, mainly manifesting as loss of vision. In addition to the development and maintenance processes of the photoreceptor itself, homeostatic processes in neighboring cells contribute to photoreceptor health. A key example of this support function is continuous phagocytosis of shed photoreceptor discs by the neighboring retinal pigment epithelium (RPE) (67, 143, 144).

Enhanced S-cone syndrome (ESCS) is a human visual disorder first recognized for its unique feature of showing increased S-cone vision. With non-invasive studies, ESCS was demonstrated to result in super-normal sensitivity to blue colors and an excess number of S-cones, normally the minority photoreceptor in the human retinal mosaic.
comprised mainly of rods and L/M-cones. The ESCS phenotype in human patients may be due to misregulation of both L and M cone progenitors, leading to an excess of S-cones expressing blue opsin, and to atypical differentiation of rods and cones (145). A hypothesis emerging from these results was that abnormal retinal development causes ESCS involving a disturbance in photoreceptor cell specification (111, 112, 146). This abnormal overexpression of S-type cones is accompanied by varying degrees of L and M cone depletion and retinal degeneration (147). A search for the causative gene(s) ensued, and most patients’ mutations mapped to the gene encoding the human photoreceptor-specific nuclear receptor, \textit{Nr2e3}, while a few mapped to the neural retina leucine zipper, \textit{Nrl}, gene (110, 148, 149). \textit{Nr2e3} and \textit{Nrl} are now known to play key roles in the regulatory transcriptional networks controlling photoreceptor cell fate (141). Identification of the causative genes, however, did not account for the degenerative component of this disease.

Knockout of the \textit{Nrl} transcription factor in mice produces a retina overpopulated with S-cone like photoreceptors along with absence of rod photoreceptors. Precise identification of changes in transcriptional networks in the \textit{Nrl}\textsuperscript{-/-} mouse retina and resulting aberrant composition of expressed proteins would likely provide information concerning critical factors that dictate cone-like photoreceptor maintenance/survival as well as proper retinal lamination. Previous studies had also suggested abnormal association between photoreceptors and the RPE in the \textit{Nrl}\textsuperscript{-/-} mouse (53, 150), and differences in RPE appearance such as discontinuity and depigmentation compared with normal RPE have been noted in human post-mortem donor ESCS retinas (113, 151).
2.2 Rationale and methodology to elucidate the degenerative component of ESCS

2.2.1 Rationale for research approach

Although ESCS was identified in humans decades ago and since then the causative genes have been elucidated, our understanding of the accompanying retinal degeneration is still poorly understood. Moreover it is important to better elucidate the genetics cues that may give rise to this abnormal retinal environment. The early stages of photoreceptor development and maintenance involve Notch signaling through basic helix loop helix (bHLH) transcription factors (152, 153) as well as through Hedgehog, which also converges on downstream Notch targets (154). The interplay of these factors, among others, dictates the proper transcriptional environment for photoreceptor maintenance, but the precise relationship between them is not yet fully elucidated. The aim of this work was to follow up on findings from human ESCS in patients in which there was a potential disruption at the photoreceptor-RPE interface. The study focused on a murine model, \( Nrl^- \) mice, that has the phenotypic features of ESCS. Through a combinatorial genetic and structural approach the aim was to improve understanding of the disease process that leads to photoreceptor degeneration and blindness, potentially guiding future therapies. The massively parallel RNA-sequencing experiment was expected to unveil new insight into the transcriptional mis-regulation in the ESCS murine model and potentially identify changes in gene expression in putative proteins involved in photoreceptor homeostasis and maintenance. The goal then was to structurally characterize wild type and ESCS murine model retinas with high-resolution imaging modalities to show that the defects leading to the progressive degenerative component of the disease were due to the inherent defect in the photoreceptors stemming from their aberrant development.
2.2.2 Materials and methods

Materials. All chemicals, unless otherwise stated, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Reagents for cDNA library preparation for Illumina sequencing, unless otherwise indicated, were bought from Illumina (San Diego, CA, USA). Reagents for cDNA synthesis and quantitative real-time PCR (RT-PCR) were obtained from Applied Biosystems (Foster City, CA, USA). Primary antibodies anti-red/green pigment opsin and anti-blue opsin were acquired from Chemicon International (Billerica, MA, USA), anti-phosphatidylserine (PS) and anti-annexinV were purchased from Abcam (Cambridge, MA, USA), peanut agglutinin (PNA) was obtained from Invitrogen (Carlsbad, CA, USA), and anti-rhodopsin was generated in the Palczewski laboratory from hybridoma cells (155). Cy3 and Alexa488 conjugated secondary antibodies were acquired from Jackson Immuno-Research (West Grove, PA, USA) or Invitrogen. Nuclear staining was achieved with Hoechst, 4',6-diamidino-2-phenylindole (DAPI) or quinolinium, 4-[3-(3-methyl-2(3H)-benzothiazolylidene)-1-propenyl]-1-[3-(trimethylammonio)propyl]-, diiodide (ToPro3) (Invitrogen).

Human studies. All ESCS patients studied had mutations in the NR2E3 gene (149). Informed consent was obtained and procedures followed the Declaration of Helsinki guidelines and were approved by the institutional review board. Patients had complete ocular examinations including kinetic perimetry quantified by published methods (156). Psychophysical thresholds were measured with a modified automated perimeter (Humphrey Field Analyzer, San Leandro, CA, USA) to determine S-cone function (440-nm stimulus on a yellow background, 170 cd m⁻²), L/M cone function (650-nm stimuli, dark-adapted) and rod function (500-nm stimuli, dark-adapted). Details of visual function
techniques and analyses have been described previously (112, 113, 146). Spectral-domain (SD) optical coherence tomography (OCT) was used (RTVue-100; Optovue Inc., Fremont, CA, USA) with published recording and analysis techniques to perform retinal cross-sectional imaging (157-160). RPE lipofuscin imaging was performed as previously described (161, 162).

Animals. Mice were housed in the animal facility at the School of Medicine, Case Western Reserve University (CWRU), where they were maintained on a standard chow diet in a 12 h light (~10 lux)/12 h dark cycle. Wild-type (Wt) mice on C57BL/6 background were obtained from The Jackson Laboratory (Bar Harbor, Maine, USA). Nrl-deficient mice in the C57BL/6 background were from Dr. Anand Swaroop (University of Michigan, Ann Arbor, MI, USA) (53). Genotyping of mice was done by PCR with primers NRL-A (5’-gtgtccttggtgggaaaga-3’) and NRL-B (5’-ctgtcacttggtggttca-3’) for Wt and NRL-KO1 (5’-tgaatacaggagacacca-3’) and NRL-KO2 (5’-ggttcataatccatcagaagca-3’) for targeted deletion of the Nrl gene. All animal procedures and experiments were performed in accordance with U.S. animal protection laws and were approved by CWRU (Cleveland, OH, USA) Animal Care Committees and conformed to both the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

Ultra-High resolution SD-OCT. Nine Wt and 9 Nrl-deficient mice aged 4 weeks were each anesthetized by intraperitoneal injection of a mixture (20 µL/g body weight) containing ketamine (6 mg/mL) and xylazine (0.44 mg/mL) in 10 mM sodium phosphate, pH 7.2, and 100 mM NaCl. Pupils were dilated with 1% tropicamide. Mice were placed in a specialized holder to permit ultra-high resolution SD-OCT (Bioptigen, Research
Triangle Park, NC, USA) for in vivo imaging of mouse retinas at $\lambda=870$ nm with a superluminescent diode. Each two-dimensional B-scan was acquired at a speed of 1000 scans per second and each final SD-OCT image was an average of 3 individual B-scans. Three-dimensional scans were taken around the optic nerve with a scanning radius of 1.6 mm. Images were post-processed by using commercial Bioptigen software and ImageJ (163).

**Library preparation for Illumina sequencing.** Mice were euthanized by cervical dislocation. Eyes were enucleated and immediately placed in RNAlater stabilization reagent (Qiagen, Valencia, CA, USA) to preserve RNA content and integrity (164) for whole eye runs. Alternatively, the retina was rapidly dissected out and similarly preserved. One mouse eye or two retinas were homogenized at once and passed through a QIAShredder column (Qiagen) as per manufacturer’s directions to further homogenize the eye tissues. Total RNA was then purified by using the RNeasy Mini Kit (Qiagen) with on column DNase treatment (Qiagen) as per manufacturer’s directions. Poly(A) RNA was isolated with the Oligotex kit (Qiagen) as per the manufacturer’s instructions. Pooled total RNA samples of 5 Wt and 5 Nrl/- female mice 4 weeks of age were used for the whole eye library preparation and pooled total RNA samples from 5 Wt and 5 Nrl/- female mice at 4 weeks of age were used for the retina library preparation.

For first strand cDNA synthesis, instructions from the SuperScript III kit protocol (Invitrogen) were followed. About 400-450 ng of isolated poly(A) RNA was mixed with 50 ng of random primers and 1 mM deoxyribonucleotide triphosphate (dNTP), incubated at 65°C for 5 min and then placed on ice for 5 min. A reaction mixture comprising 5 mM MgCl$_2$, 10 mM DTT, 40 U RNaseOUT, and 200 U SuperScript III reverse transcriptase
was added to the initial mix to achieve a total volume of 20 µL. The mixture was incubated at 25°C for 10 min, followed by 50 min at 50°C. The reaction was terminated at 85°C for 5 min and then chilled on ice for 10 min. At this point, 2 U RNase H was added and the mix was incubated at 37°C for 20 min. The first strand cDNA synthesis reaction was immediately used for second strand synthesis. To the first strand product, 300 µM dNTP, *E. coli* DNA polymerase I buffer, and water was added to obtain a total volume of 95 µL and allowed to incubate on ice for 10 min. Then, 0.05 U *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA, USA) was added and the mixture was incubated at 16°C for 2.5 h. The resulting double stranded cDNA was purified with the Wizard SV Gel and PCR Clean-up System (Promega, Madison, WI, USA) and eluted in 100 µL nuclease-free water and then fragmented by the Covaris S2 instrument (Woburn, MA, USA) to generate ~200 bp fragments as follows: 10% duty cycle, intensity of 5, 100 cycles per burst, with a bath temperature of 7.7°C and an acoustic power of 24 W.

The Illumina library was prepared according to the manufacturer’s instructions and purified using the Wizard SV Gel and PCR Clean-up System (Promega). Overhangs were converted into blunt ends with T4 DNA polymerase and Klenow DNA polymerase by incubating the mixed sample at 20°C for 30 min. cDNA was purified and eluted in 32 µL of nuclease free water with the Wizard Plus Minipreps DNA purification system (Promega). The purified sample was then mixed with Klenow fragment (3’ to 5’ exo minus) and incubated at 37°C for 30 min to add an ‘A’ base to the 3’ end of the blunt phosphorylated DNA fragments. The cDNA was then purified and eluted in 23 µL of nuclease free water with the Wizard Plus Minipreps DNA purification system (Promega). Eluted DNA was mixed with Illumina Adapter Oligo mix and T4 DNA ligase and
incubated at room temperature for 15 min to ligate adapters to the ends of the DNA fragments to prepare them for hybridization to the flow cell. cDNA then was purified and eluted in 10 µL of nuclease free water with the Wizard Plus Minipreps DNA purification system (Promega). cDNA templates were purified by running samples on a 1% agarose gel at 100 V for 60 min and excising the region of the gel in the 200 bp range. The 200 bp cDNA enriched fragments were purified and eluted in 30 µL of nuclease free water with the Wizard Plus Minipreps DNA purification system (Promega). cDNA in the library was then amplified by a 15 cycle PCR with two primers that annealed to the ends of the adapters. The amplified cDNA was purified and eluted in 30 µL of nuclease free water with the Wizard Plus Minipreps DNA purification system (Promega). The size, purity and concentration of the final library was checked with the Bio-Rad Experion DNA specific chip prior to sequencing by using the Illumina Genome Analyzer. The concentration of the sample was also measured using 1 µL of purified sample with the Qubit Quantitation Platform (Invitrogen) to estimate loading conditions for the Illumina Cluster Station.

**Illumina Genome Analyzer II RNA-Seq runs, read mapping, and FPKM determination.** Each library was run on three lanes of the Genome Analyzer II in the Genomics Core Facility at CWRU by using 36 or 49 base pair (bp) single-end sequencing. The numbers of mapped single reads from different experiments were 381,661,42 from the whole eye technical replicates; 45,431,330 from the Wt whole eye; 66,643,381 from the Nrl<sup>-/-</sup> eye; 85,159,191 from the Wt retina; and 104,081,398 from the Nrl<sup>-/-</sup> retina. Technical replicates of the whole eye entailed running the sample library preparation on independent lanes on different day runs and analyzing them separately.
Primary data transformation included image analysis, intensity scoring, base calling and alignment, all carried out with Illumina pipeline software running on Linux. Image analysis identified distinct clusters and created digital intensity files describing the signal intensity of each cluster per cycle. Signal intensity profiles for each cluster were used to call bases and quality scores for each base call were calculated for alignment. Efficient Large-Scale Alignment of Nucleotide Data-bases (ELAND) (Illumina) was then used for read mapping to the UCSC mouse genome assembly and transcript annotation (mm9)(165). For each read, ELAND determined the position in the genome to which the read substrings matched with a maximum of two errors. Base quality scores and the positions of the mismatches in a candidate alignment were used to calculate a probability score for each candidate, with the highest probability score indicating the best candidate. Eligible reads were defined by having a unique alignment to the genome or a single most probable alignment to the genome. Other reads with failed quality control measures were not used in subsequent processing. The ELAND alignment was loaded onto Consensus Assessment of Sequence and Variation (CASAVA) (Illumina) software for calculation of fragments per kilobase of exon model per million mapped reads (FPKM) statistics by gene, transcript, and exon. CASAVA counted the number of bases that belong to exons and genes and the number of bases that fall into the exonic regions of each gene was summed to obtain gene level counts. Normalized values were then calculated as FPKM. The output for CASAVA was visualized with the GenomeStudio RNA Sequencing Module (Illumina), which allowed comparison between the samples based on the CASAVA output files.
**Real time-polymerase chain reaction (RT-PCR).** Two µg of isolated total retinal RNA from 3 pooled Wt and Nrl⁻/⁻ samples were converted to cDNA with the High Capacity RNA-to-cDNA kit (Applied Biosystems). RT-PCR was done with TaqMan chemistry and Assays on Demand probes (Applied BioSystems) for mouse Abca4 (Mm00492035_m1), Atp8a2 (Mm00443740_m1), Atoh7 (Mm00844064_s1), Bmp15 (Mm00437797_m1), Crx (Mm00483995_m1), Egr1 (Mm00656724_m1), Eya1 (Mm00438796_m1), Gdf11 (Mm01159973_m1), Neurod1 (Mm01946604_s1), Notch1 (Mm00435249_m1), Prdm1 (Mm01187284_m1), Opm1sw (Mm00432058_m1), Otx2 (Mm00446859_m1), Six6 (Mm00488257_m1), Six6os1 (Mm01290652_m1), Thrb (Mm00437044_m1), Rxrg (Mm00436411_m1), and Wnt9b (Mm00457102_m1). The 18S rRNA (4319413E) probe set (Applied BioSystems) was used as the endogenous control. All real-time experiments were done in triplicate with the ABI Step-One Plus qRT-PCR machine (Applied BioSystems). Fold changes were calculated based on differences in threshold cycles (Ct) between the Nrl⁻/⁻ and Wt samples after normalization to 18srRNA.

**Analysis of transcriptome data.** Fold-differences in RNA-Seq experiments were compared by examining the ratio of FPKM between Wt and Nrl⁻/⁻ sample runs. A 1.5-fold or greater change in threshold was used to identify differential expression thereby allowing comparisons with previous experiments. Statistical significance of fold expression changes in RT-PCR were analyzed with Microsoft Excel software. P-values were calculated from a student’s two-tailed t-test to confirm that fold changes were statistically significant (p-value < 0.05). Power analysis was calculated to detect the sample size required to detect significant changes with RNA-Seq using a 1.5-fold difference cut-off. The parameters were detecting a 0.33 FPKM difference (a 1.5
decreased fold of 1 FPKM, representing an expressed transcript, is 0.67, yielding a difference of 0.33 FPKM), a standard deviation of 10% in the FPKM value (estimated from technical replicates), an alpha value of 0.05, and a beta value of 0.10, with the ratio of Wt to Nrl\(^{-/-}\) samples as 1.

**Cryo-sectioning.** Twenty Wt and 20 Nrl-deficient mice aged 4 weeks were sacrificed 1.5 h after lights went on in the morning, a time when phagocytosis of photoreceptor OS in Wt is maximal. Eye cups were dissected out under a surgical microscope and incubated in 4% paraformaldehyde overnight at 4°C. Eye cups then were dehydrated in successive solutions of 5%, 10%, 15%, and 20% sucrose in phosphate buffered saline (PBS: 137 mMNaCl, 2.7 mM KCl, 4.3 mM Na\(_3\)HPO\(_4\), 1.4 mM KH\(_2\)PO\(_4\), pH 7.3) for 30 min each on a shaker. Subsequently, eyes were placed in a 1:1 solution of 20% sucrose in PBS: Optical Cutting Temperature Compound (Tissue-Tek-Sakura, Torrence, CA, USA) for 30 min on a shaker, when the solution was replaced and the eye cups were kept at 4°C overnight. Eye cups were frozen the next day by placing them in cryo-molds and submerging them into 2-methyl-butane in a tank of liquid nitrogen. Cryo-blocks were then cut with a Leica cryo-sectioner and 10 \(\mu\)m sections around the optic nerve were collected on glass slides for immunohistochemical staining.

**Immunohistochemistry.** All procedures used were reported previously (166, 167). Cross-sections of mouse eyecups were incubated with primary antibodies, namely anti-rabbit red/green pigment opsin, anti-rabbit opsin blue anti-mouse rhodopsin, anti-mouse PS, anti-rabbit annexin V, and PNA. Signals were detected with either Cy3-conjugated secondary antibody or Alexa488 conjugated secondary antibody. Nuclear staining was
achieved with DAPI. Sections were analyzed with a Leica 6000B microscope (Leica, Wetzlar, Germany).

**Scanning electron microscope (SEM).** Seven Wt and 9 Nrl-deficient mice 4 weeks of age were sacrificed and their retinas and the RPE was separated and fixed in 2.5% glutaraldehyde, 0.1 M cacodylate buffer, 2% sucrose, pH 7.4 for 24 h. Samples were washed in 0.1 M cacodylate buffer and 2% sucrose, fixed with 1% OsO₄ in washing buffer, dehydrated with ethanol, dried by a critical point drying method (168), and sputter coated with a 5-10 nm thick gold layer. Samples were imaged with a JSF-6300F SEM (JEOL, Japan) at the University of Washington, Department of Pathology, Seattle. The emission current was set to enable acquisition of backscattered electron scanning images between 2000X and 10,000X magnification.

**Transmission electron microscopy (TEM).** Five Wt and 5 Nrl-deficient mice aged 4 weeks and 5 Wt and 5 Nrl-deficient mice aged 8 weeks were sacrificed 1.5 h after lights went on in the morning. Eyes were removed and whole eye cups were dissected out under a surgical microscope and placed in 4% paraformaldehyde at 37°C for 4 h. Eye cups then were rinsed in PBS and incubated in a 1:1 solution of 2% OsO₄:3% potassium ferrocyanide for 1 h. This was followed by incubation in a new mixture of 2% OsO₄:3% potassium ferrocyanide for 1 h after which eye cups were washed in filtered water and placed in 0.25% uranyl acetate overnight at 4°C. Eye cups were dehydrated the next day for 10 min each in sequential solutions of 30%, 50%, 75%, 85%, 95%, and 100% ethanol in water, then for 15 min each in sequential solutions of 50%, 75%, and 100% propylene oxide in ethanol followed by 2 h in 30% epon in propylene oxide and finally kept in 50% epon in propylene oxide overnight. Next day eye cups were placed in 75% epon in
propylene oxide for 4 h, then in 100% epon for 2 h under vacuum and finally in a mold with epon kept at 73°C for 4 days to enable cross-linking. Then blocks were cut with a microtome and ultra-thin sections (0.07 µm) were stained with uranyl acetate and adsorbed onto carbon grids. A Tecnai T12 EM (FEI, Eindhoven, Netherlands) operating at 120 kV with a tungsten filament was used for final imaging.

**Eye tissue preparation, sectioning and imaging for focused ion beam (FIB)-SEM experiments.** Wt and *Nrl*−/− mice at age 4 weeks were sacrificed and eye cups were dissected under a surgical microscope. The cornea was cut and lens removed. Retinas and retinal pigmented epithelium (RPE) layers were carefully separated. Tissues were then fixed in a solution of 2.5% glutaraldehyde, 0.1 M cacodylate buffer, 2% sucrose, pH 7.4, for 24 h at 4 °C. Samples were washed the next day in 0.1 M cacodylate buffer, 2% sucrose, pH 7.4, and subsequently fixed in 1% OsO4 in 0.06 M cacodylate buffer, 5% sucrose, pH 7.4, for 2 h. The samples were then washed and dehydrated with ethanol and dried by a critical point drying method (168). The retina and RPE were separately mounted on standard SEM stubs with carbon tape and the edges covered with silver paste as needed and stored overnight at 30 °C. The cured samples were finally sputter-coated with a 5-10 nm thick gold layer. The prepared retina or RPE sample was placed inside an xT Nova Nanolab 200 or Helios Nanolab 650 (FEI, Eindhoven, The Netherlands) dual beam microscope system fitted with a field emission gun for scanning electron imaging and a focused ion beam of gallium ions for milling. Once the area of interest was identified, a ~1-2 µm thick layer of platinum (Pt) was deposited at ion currents ranging from 1 to 7 nA by a gas injection system using a Pt organometallic vapor specie. The current used to layer Pt was dependent on the experiment. In cases where cells were
tightly packed and were sufficiently sputter coated that there were not empty patches, Pt was deposited at high current (3-7 nA) in a short time (1-5 mins) to coat the area of interest. In cases where the cells were not sufficiently packed and thus exhibited uneven sputter coating, Pt was deposited at lower current (0.4-3 nA) over a longer time (15-30 mins) to fill in the empty patches and generate an even Pt coat over the area of interest.

The imaging face was prepared by setting up a cleaning cross section using the FIB at 30 kV. To allow imaging of the internal architecture with the electron beam, a trench was created in front of the Pt protected region of interest by milling away with the ion beam at currents ranging from 7 to 30 nA. The milled face was larger in both x and y directions than the area to be imaged, to prevent any ion deposition that could interfere with imaging. Once the trench was milled to the edge of the first protected sample face to be imaged, a 0.2 µm high fiducial pad was patterned at a current of 0.79 nA away from the field of interest to provide a landmark for microscopic alignment before each cut.

Image collection parameters were set up using ‘slice-and-view’ G2 and G3 imaging software (FEI). Ion beam parameters were set to mill layers from the region of interest, i.e., 35-50 nm thick for these experiments. Different milling sizes were achieved by varying the deflection of the ion beam. After each layer was removed, a secondary electron image was collected in the immersion lens mode. Secondary electron or backscatter electron scanning images were typically recorded at accelerating voltages of 2 to 3 kV and detected via either the in-lens detector or the ETD detector in the field free mode. Images were collected with a dwell time of 10-30 µs/pixel at a resolution of 4096 x 3536 pixels. For each image collected, the contrast was adjusted automatically. Milling and image acquisition time for each section averaged ~4 min. A typical experiment
ranged from 100-500 collected slices over 6 to 30 h of acquisition time. Automation and drift adjustment allowed collection of serial sections overnight without any operator present. Acquired SEM images were opened with Fiji-win32 (free for use released under the General Public License) and merged to form a stack. The stack was registered by using a rigid feature extraction model that accounted for translation and rotation of sections over the time course of the experiment. The software extracted key objects from a set of reference images and found candidate matching features based on Euclidean distance of their feature vectors over the time course of experiment. In cases where the collected image files were too large to open in Fiji-win32 running at full memory capacity, stacks were virtually registered. Registered images were saved and then imported into the Reconstruct program (169) and the distance between each section was specified.

Individual photoreceptors and their disc elements were mapped in Reconstruct. With the ‘Autotracing’ tab in ‘Series Options’, the pixel intensity values were adjusted in the ‘Wildfires’ tools window for the program to recognize the boundaries of each photoreceptor disc. Once these parameters were set (these varied for each experiment, based on brightness and contrast), the Wildfire tool was used to fill in each individual disc and subsequently render it in 3D. Individual photoreceptor cell outlines were manually traced by hand because the sensitivity of the Wildfire tool did not always allow contrast distinction between cells.

**SBF-SEM, SEM data analyses and 3D reconstruction.** The same blocks prepared for TEM were used for SBF-SEM (serial block face scanning EM) (170). To prepare a sample, we used an ultramicrotome (Leica UCT) and a diamond knife (Diatome,
Hatfield, PA, USA), and trimmed the block so that only resin embedded tissue of the region of interest remained. The final tissue block was adhered by conductive carbon cement to an aluminum SEM stub to preserve conductivity. The prepared sample was fixed on the microtome (3View, Gatan, Pleasanton, CA, USA) attached on the door of the SEM (QUANTA 200 FEG ESEM, FEI). Cutting was initiated in the evacuated specimen chamber. To perform serial cutting of the block face, a 100 nm slice was cut from the face with a diamond knife and the freshly cut surface of the block was imaged from the backscattered electron signal. This process was repeated sequentially in an automatic computer-controlled fashion to collect 500 successive images over ~12 h. Imaging was performed at an accelerating voltage of 3 kV in a low vacuum mode (0.23 Torr) at 4096x4096 pixel resolution at a rate of 3 µs/pixel.

After serial sectioning, images were opened with Fiji-win32 (a version of ImageJ, http://imagej.nih.gov/ij/index.html) and merged to form a stack. The stack was registered and aligned to account for any drift that may have occurred over the time course of sectioning. The registered stack then was opened using the Reconstruct program (171) and structural elements were mapped to provide three-dimensional reconstructions.

**Whole mount retinal confocal microscopy.** Ten Wt and 10 *Nrl*-deficient 4-week-old mice were sacrificed and eye whole mounts were prepared and incubated overnight with primary antibodies, *i.e.* anti-rabbit red/green pigment opsins, anti-rabbit opsins, anti-mouse rhodopsin, anti-mouse PS, and PNA. Signals were detected with either Cy3-conjugated secondary antibody or Alexa488 conjugated secondary antibody. Nuclear staining was achieved with ToPro3. Thirty- to 40-µm thick z-stacks were collected at 40x magnification with 1 µm between each slice and visualized with a Leica SP5 confocal
microscope. Obtained images were post-processed with ImageJ to adjust contrast and brightness.

**Phagocytosis assays of RPE cell cultures**

RPE was isolated from 10 to 12 day old Wt and *Nrl*−/− mice as previously described (172). Briefly, eyes were removed from animals and washed twice in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with non-essential amino acids. Eyes were incubated in 2% dispase (Invitrogen) solution for 45 min in a 37°C water bath with occasional tube inversion. Eyes were washed twice in cold DMEM plus streptomycin/penicillin (Invitrogen), 10% fetal bovine serum (Invitrogen), and 20 mM HEPES pH 7.2. Eyes were enucleated and the cornea, lens and iris were removed. Eye cups were incubated in DMEM plus streptomycin/penicillin, 10% fetal bovine serum, and 20 mM HEPES, pH 7.2, in a 37°C incubator for 15 min to facilitate removal of the neural retina. After removal of neural retina, sheets of continuous RPE were peeled from choroid and pipetted into a tube containing DMEM plus streptomycin/penicillin and 10% fetal bovine serum. RPE sheets were subsequently filtered over a 40 μm cell strainer (Fisher Scientific, Pittsburgh, PA, USA) to remove contaminating cell types. Sheets were spun at 200×g for 3 min and then re-suspended in DMEM plus streptomycin/penicillin and 10% fetal bovine serum before gentle disruption by pipetting. Disrupted cells were seeded onto 24-well 0.4 μm transwell permeable supports (Corning Incorporated, Corning, NY, USA) with the RPE from ~ 2 eyes per well to allow polarization of cells. Cells were grown for 5-6 days at 37°C, 5% CO₂ before use in phagocytosis assays.

For phagocytosis challenge assays, photoreceptor OS membranes were isolated from Wt and *Nrl*−/− mice. Photoreceptor outer segment membranes from Wt were isolated
as previously described (173) whereas OS membranes from \( Nrf^{+/} \) were obtained by a similar protocol with 10-100% continuous gradient of OptiPrep (Nycomed, Norway) to improve the yield. Photoreceptor OS membranes isolated from Wt and \( Nrf^{-/-} \) mice were covalently labeled with fluorescein isothiocyanate (FITC) (Invitrogen) by using established protocols (174). FITC-labeled photoreceptor OS membranes were resuspended in DMEM plus streptomycin/penicillin, 10% fetal bovine serum, 2.5% sucrose and 50 µL of this mixture was added to top of the transwell membrane while 700 uL of DMEM alone plus streptomycin/penicillin, 10% fetal bovine serum was added to the well of the plate. Assay mixtures were incubated in the dark at 37°C for 1 h. Cells were washed 3 times with PBS plus 1 mM MgCl\(_2\) and 0.2 mM CaCl\(_2\) (PBS-MC). FITC fluorescence of externally bound photoreceptor OS was quenched by incubation with 0.2% Trypan Blue (Invitrogen) for 10 min, after which cells were washed 3 times with PBS-MC. Cells were fixed with ice cold methanol for 5 min at 4°C followed by 3% paraformaldehyde at room temperature for 10 min. Cells were washed 2 times with PBS-MC and permeabilized with 0.2% Triton X100 in PBS for 30 min at room temperature. Nuclear staining was performed by incubation with Hoechst stain (10 µM final) for 30 min at room temperature. Cells were washed in PBS-MC an additional 3 times. Transwell membranes were removed from supports and mounted onto microscope slides with ProLong Gold Antifade Agent (Invitrogen).

2.3 Results

2.3.1 Phenotypic features of human ESCS patients and the relationship of these features to the \( Nrf^{+/} \) mouse model
The diagnosis of ESCS is based on a quantitative comparison of S-cone and L/M-cone visual and/or retinal parameters (112, 146, 175, 176). Normally, L/M-cone vision is far more sensitive than S-cone vision, but in ESCS it is surprisingly just the opposite. ESCS manifests heightened sensitivity of S-cone vision relative to L/M cone vision in the presence of little or no rod function. Comparison between a 13-year-old boy with ESCS (Patient 1, P1) and a normal subject exemplified the increased S-cone function and reduced L/M-cone vision compared with results in a normal subject (Figure 11A). The sensitivity difference was positive (Figure 11A, third column: ‘Enhanced S-cone function’ denoted by + symbols at test loci) in contrast to negative numbers when normal L/M-cone sensitivities were subtracted from S-cones (112). P2, another ESCS patient at two different ages (40 and 48 years) showed the same diagnostic difference at loci with persistent function despite reduced S- and L/M-cone vision because of progressive retinal degeneration (Figure 11A). Progressive degenerative retinopathy of ESCS was further illustrated by plotted kinetic visual field data from 9 ESCS patients followed longitudinally for at least a decade (Figure 11B). Relatively full visual fields tended to become reduced with age, leaving only central and peripheral islands separated by blind spots (Figure 11B, insets).

In vivo histopathology in early stages of ESCS showed a hyper-thick photoreceptor outer nuclear layer (ONL) in the more central retina but a variably reduced ONL with increasing retinal eccentricity (Figure 11C). In the extra-central retina of ESCS patients, there was noticeable dysmorphology of the ONL with intraretinal hyper-reflective lesions extending to the inner retina (for example, in P1). Longitudinal reflectivity profiles of the outer retinal laminar architecture in two normal subjects at 2.5 mm from the fovea
showed layers of ONL, photoreceptor inner segments (IS), rod OS, cone OS and RPE (Figure 11D). Three ESCS patients (ages 17, 13, 31, left to right) had a noticeably thickened ONL (177) and appear to have a thickened IS layer as well. When the normal IS layer thickness (n=6 ages 8-29; mean ± 2SD, 27±2.8 µm) was compared with IS thickness in 6 ESCS patients (ages 13-31; mean ± 2SD, 35±6.7 µm), the IS layer in ESCS patients was significantly thicker (t-test, p<0.001). This may relate to the longer IS in human S-cones seen in morphological studies (178). The interface between photoreceptor OS and RPE was also abnormal and ill-defined, i.e. the normal stereotypical multi-peaked profile is not evident in ESCS patients (Figure 11D). The reason for the abnormal interface between photoreceptors and RPE found in these imaging studies is not known. *En face* imaging further illustrated abnormalities in ESCS patients. In normal subjects, autofluorescence (AF) emissions upon short wavelength excitation are dominated by spatially homogeneous lipofuscin granules accumulated in the RPE (162, 179), but ESCS patients exhibited hyperautofluorescent loci in the macular and midperipheral retinal regions. Cross-sectional imaging of co-localized regions showed dysmorphology of the ONL extending to the inner retina (Figure 11E, insets; dysmorphology also seen in temporal retina of P1, Figure 11C). Abnormal deposition of retinal or RPE fluorophores, unmasking of natural fluorophores by localized loss of RPE melanin, or lipofuscin-laden macrophages, alone or in concert, could contribute to these hyperautofluorescent features (180-182).

The *Nrl*−/− mouse exhibited many phenotypic features of human ESCS disease. Given that ESCS is a rare Mendelian disease, this mouse model provided an opportunity to study ESCS pathophysiology in a more systematic way in large numbers. Retinal
degeneration is not yet evident at 4 weeks of age in the Nrl−/− murine eye. However, cross-sectional optical imaging by spectral domain optical coherence tomography (SD-OCT) revealed abnormal retinal lamination in the Nrl−/− compared to the Wt retina (Figure 11F). Three-dimensional reconstructions allowed visualization of abnormal intraretinal hyper-reflective lesions, presumed to be rosettes, and how this distortion affected the retinal laminar architecture (Figure 11G). Plastic block and cryo-sectioning of retinas further highlighted the dynamic changes resulting from the excessive S-cone photoreceptor population at higher resolution. Compared with normal Wt retina (Figure 12A-D), the Nrl−/− retina displayed aberrant photoreceptor packing and abnormal association with the RPE (Figure 12E-H).

Although these data demonstrated comparable progressive retinal degenerative features in human and the mouse models of ESCS, the fundamental cellular aberrations that cause this pathophysiology are unknown. The approach was to unveil the molecular basis of this degeneration with a comprehensive global sequencing approach.

2.3.2 Transcriptome analysis by Illumina based RNA-Seq of retinas from Wt and Nrl−/− mice

By sequencing mature Wt and Nrl−/− ocular tissues, global changes resulting from knockout of the Nrl transcription factor could be determined. The reproducibility of RNA-Seq for murine eye was verified by carrying out technical replicates of Wt eye samples (R²=0.98, Figure 13A), indicating there was minimal variability from run to run. This reproducibility indicated any differences between different tissue samples were not inherent to instrument read errors. Wt retinal tissue (Figure 14A) generated 11,677 unique transcripts at a level of 1 FPKM or higher (Figure 14B) whereas Nrl−/− retinal
tissue (Figure 14C) generated 11,778 unique transcripts at a level of 1 FPKM or higher (Figure 14D). In Wt and Nrl⁻/⁻ retinal tissues, a large proportion of transcripts had no annotated function. A complete categorization of GO terms from these tissues is shown in Table 1. RNA-Seq expression analysis on whole eye tissues of Wt and Nrl⁻/⁻ mice is summarized in Figure 13C-F. The RNA-Seq findings revealed that, across experiments, the number of transcripts detected between runs was not drastically different nor was their categorization, indicating there is not a gross difference between Wt and Nrl⁻/⁻ retinas and eyes, but rather more subtle differences that require RNA-Seq single gene resolution to tease out their precise differential expression patterns.

2.3.3 Verification of sequencing data by RT-PCR

Differential expression of transcripts was analyzed by comparing FPKM values between Wt and Nrl⁻/⁻ retinal (Figure 14E) and whole eye (Figure 13B) tissues. Previous microarray studies had used an empirical cut-off of a 1.5-fold average change as the minimum to identify a difference between the Wt and Nrl⁻/⁻ genotypes (55). Using this same cut-off, 7316 and 6203 differentially expressed transcripts were identified in whole eye and retinal samples, respectively. Previous studies have used in-situ hybridization and RT-PCR to verify results of microarray analyses (55, 131), results of which overlapped with our current RNA-Seq data (Figure 13). Therefore, RT-PCR was carried out to verify differentially expressed transcripts identified by RNA-Seq. Compared with the 66 transcripts that were differentially expressed and overlapped with findings of all three studies, RT-PCR had been previously done with 31 of these targets (55), and the RNA-Seq data presented here indicated an excellent correlation of fold differences.
between Wt and Nrl−/− retinal tissues for these 31 targets (R²=0.91). RT-PCR on targets were chosen representing a new signaling pathway that may dictate the formation and maintenance of the cone-line environment in the Nrl−/− retina. Transcripts with large differential expression (Egr1, Opn1sw), more subtle changes (Gdf11, Otx2, Thrb) and no significant change (Crx) were used. The results of the RT-PCR strongly correlated with the differential expression detected by RNA-Seq analyses of the retina and whole eye (Figure 14F). Given the precise quantification of the transcript levels, it was calculated that the signal needed for detecting a fold change of 1.5 with statistical significance required only 2 biological samples. Additionally, because this trend in differential expression was preserved in RNA-Seq runs of the whole eye and retina (Table 2), this also indicated that the detected unique transcripts truly represent significant changes between the Wt and Nrl−/− genotypes. Therefore, a thorough analysis of the murine retina transcriptome was well warranted.

2.3.4 Characterization of differentially expressed transcripts

Direct comparison of previous retinal microarray studies (55, 131) with the RNA-Seq of the Wt and Nrl−/− retina revealed that of the 6203 differentially expressed transcripts, 5889 were unique to this study with 3659 of the transcripts up-regulated (Figure 14G) and 2230 transcripts down-regulated (Figure 14H) in the Nrl−/− retina. The greater number of up- as compared to down-regulated transcripts using the same thresholds is similar to what was reported in previous microarray experiments (131). Breakdown of transcripts by their fold change revealed that RNA-Seq identified more subtle changes in transcript levels than microarrays (Figure 14G-H).
Examination of transcripts with large differences between Wt and \( Nrl^{-/-} \) expression revealed 248 transcripts to differ by 5-fold or more, of which 134 were unique to this study. Pathway analysis was done for all 248 transcripts. In particular, analysis of the 134 unique transcripts identified several that were critical in pathways involved in photoreceptor differentiation and maintenance, such as atonal homolog 7(\( Atoh7/Math5 \)), a bHLH factor involved in Notch signaling (183); \( \text{Six6} \), a sine oculis-related homeobox gene in the pathway of the master regulatory genes of eye development, \( \text{Pax6} \) and \( \text{Eya1} \) (2, 3, 184-188); and desert hedgehog (\( \text{Dhh} \)), a sonic hedgehog signaling molecule (189, 190). Furthermore, several key developmental maintenance pathways such as the Wnt (191, 192) and Bmp signaling pathways (193, 194) had mis-expressed transcripts in the \( Nrl^{-/-} \) retina such as \( \text{Wnt9b} \) and \( \text{Bmp15} \). Pathway analyses of these transcripts, coupled with other transcripts found in this and previous studies to be mis-regulated, provide a more comprehensive transcriptional landscape to examine S-cone commitment and maintenance in the \( Nrl^{-/-} \) mouse retina. Notably, 29 out of 134 transcripts had no annotated function and thus represented new targets for study of cone-like photoreceptor maintenance and function.

Examination of homeostatic processes involved in photoreceptor function in the visual cycle revealed cone signature genes (\( \text{Pde6c} \) and \( \text{Cngb3} \), among others). It also revealed down-regulated genes important for retinoid metabolism and clearance of potentially toxic photo-oxidized compounds (\( \text{Abca4}, \text{Rdh12} \) and \( \text{Rdh5} \) among others) (Table 3). In contrast, expression of genes encoding putative proteins involved in RPE-mediated phagocytosis responsible for toxic metabolite removal and re-cycling were unchanged. However, key photoreceptor ligands necessary for phagocytosis, \( \text{Tub} \) and
*Tulp1* (195), were down-regulated in the *Nrl*⁻/⁻ retina (*Table 4*). Down regulation of key retinoid metabolic genes, coupled with down-regulation of *Tub* and *Tulp1*, suggested a potential mechanism involving defective phagocytosis underlying the photoreceptor degeneration seen in ESCS. Therefore, to verify a potential aberrant phagocytic process, the phenotypic impact of the differential expression changes identified by RNA-Seq were investigated by high-resolution imaging studies.

2.3.5 Disrupted ESCS retinal architecture and patchy loss of photoreceptors

Overproduction of short wavelength-sensitive photoreceptors in ESCS patients causes retinal disorganization and has been analyzed only by conventional optical methods. The *Nrl*⁻/⁻ mouse retina offers the possibility to investigate the structural phenotype in greater detail. Confocal microscopy imaging of *Nrl*⁻/⁻ mouse retinal whole mounts dramatically illustrated disrupted architecture in three-dimensional space (*Figure 15*). When compared to cone-like photoreceptor packing in Wt mouse retina (*Figure 15A-B*), *Nrl*⁻/⁻ retina exhibited aberrant clustering of photoreceptors with empty patches where nuclear rosettes had formed (*Figure 15G-H*). Wt retinal whole mounts stained for both rods and cones displayed the entire area populated by photoreceptors (*Figure 15C-D*), whereas *Nrl*⁻/⁻ retinal mounts featured irregular photoreceptor packing (*Figure 15G-H*). Notably, *Nrl*⁻/⁻ photoreceptor density was clearly reduced as indicated by empty patches of retina that lacked photoreceptor staining (*Figure 15I-J*).

To probe the photoreceptor morphology, critical point dried retinas separated from the RPE of both Wt and *Nrl*⁻/⁻ 4-week-old mice were prepared. As noted from previous work (173), scanning EM (SEM) imaging revealed that Wt retina contained tightly packed, cylindrically-shaped rod photoreceptors (*Figure 15E-F*). In contrast, *Nrl*⁻/⁻
photoreceptors exhibited a different structure and packing arrangement. In agreement with whole mount confocal microscopy imaging, SEM imaging showed patches of ESCS photoreceptors clustered together and separated by patches devoid of photoreceptors (Figure 15K). When these photoreceptors were probed at higher magnification, their OS appeared bulbous (Figure 15L). This abnormal phenotype could explain why the packing density of photoreceptors was greatly reduced in ESCS (n=5, 0.38±0.04 photoreceptors/square micron) relative to Wt retina (n=5, 0.86±0.06 photoreceptors/square micron) when analyzing the SEM data. To better understand the structural defect contributing to this bulbous OS phenotype in Nrl−/− photoreceptors, thin sections of photoreceptors by transmission EM (TEM) were analyzed to examine their internal architecture.

2.3.6 ESCS photoreceptors exhibit abnormal accumulations of material

Thin sections of retina were prepared and examined by TEM. Wt rods displayed neatly stacked discrete discs (Figure 15M-N). Because these blocks were prepared shortly after the onset of light, coinciding with the peak of OS disc shedding (143), imaging of the photoreceptor-RPE interface also revealed phagosomes that dispose of shed OS disc membranes (Figure 15O). TEM imaging of photoreceptors in Nrl−/− retina showed an OS disc arrangement distinctly different from rods (Figure 15P), with some discs exhibiting interconnections to each other and the surrounding plasma membrane (Figure 15P-Q). There also was an abnormal build-up of material in ESCS photoreceptors at the photoreceptor-RPE interface (Figure 15R). OS tips of these photoreceptors were enlarged because of an internal build-up of vacuole-like structures. By 8 weeks of age, when degeneration is present, thin sections of Nrl−/− retina revealed photoreceptor OS that
exclusively contained vacuole-like structures with only a few disc elements present (Figure 16). This abnormal accumulation of material was investigated using high-resolution FIB-SEM techniques to identify in more detail this aberrant morphology.

2.3.7 Aberrant distribution of disc membranes influences abnormal packing architecture of ESCS photoreceptors

Preparation of Wt mouse retina, which is predominantly populated by rod photoreceptors, allowed structural analysis of these specialized neuronal cells. Wt samples were first prepared and analyzed to provide a baseline for these experiments as they had yet to be carried out using retinal tissue. By varying the magnification and step size of the FIB-SEM ion beam cuts, different structural aspects of rod photoreceptors were examined. The first set of experiments entailed examining the Wt mouse retina sample at lower magnifications to discern the overall arrangement of individual rod cells and their packing. The created trench in front of the area of interest allowed visualization of the internal architecture of the rod photoreceptors (Figure 17A) and revealed the intimate packing of these cells next to each other (Figure 17B). The SIA serial sectioned data set was loaded into Fiji-win32 for stack alignment and registration. The data was then loaded into Reconstruct for three-dimensional reconstruction of individual photoreceptor cells (Figure 17C). The FIB-SEM data and subsequent reconstructions revealed that the rod photoreceptors had diameter of 1.2 µm ±0.1 µm, in agreement with previously published data (34, 173, 196).

To better examine the internal photoreceptor disc architecture, rod photoreceptors were imaged at higher magnification with more precise ion beam cuts (35 nm) to accurately identify and reconstruct the discs. Because rod photoreceptors of a critical
point-dried sample do not form an even surface (Figure 18A), the area of interest was protected with a thick layer of deposited platinum (Figure 18B) to minimize milling artifacts such as excessive streaking or vertical stripes along the block face known as curtaining (197). Again, the trench created allowed visualization of the internal disc elements of rod photoreceptor outer segments (Figure 18C). Moreover, at this resolution, one could examine the rod cell and view the intimate internal disc structures (Figure 18D) and three-dimensionally reconstruct a portion of the rod outer segment complete with internal disc structures (Figure 18E). The reconstructed discs had a diameter of 1.1 μm ±0.3 μm (n=41 discs), in agreement with previously published data (34, 173, 196).

After examining rod photoreceptors, this technique was applied to Nrl−/− mice to understand the structural changes accompanying the ESCS phenotype. Nrl−/− mouse retinas were similarly prepared as Wt mouse retinas and analyzed by FIB-SEM. Initial imaging of Nrl−/− photoreceptors highlighted an abnormal phenotype wherein the photoreceptor outer segments adopted a bulbous appearance (Figure 19A). These photoreceptors did not exhibit tight packing, so platinum application for even milling was done at low current (0.4 nA) for longer periods (15-30 min) to get an uniform coating (Figure 19B). To visualize the Nrl−/− photoreceptor outer segments (Figure 19C), an ion-beam cleaning cross section pattern was used. The photoreceptors displayed aberrant packing, but even more striking was the disorganization of the internal architecture of the outer segments (Figure 19D). Three-dimensional reconstructions of the sections (Figure 19E) revealed that whereas the discs near the photoreceptor inner segment retained normal morphology, and had some connections to the photoreceptor membrane akin to normal S-cones, the photoreceptor outer segment head exhibited a collection of
disorganized structures that could explain the abnormal outer segment phenotype. The disorganized structures ranged in size from 0.4-0.8 μm, and thus most likely do not represent normal photoreceptor disc membranes.

Moreover, this disruption in disc membrane architecture serves to disrupt the packing of the photoreceptors in the retina. When looking at overall photoreceptor architecture (Figure 20A-B), the results for photoreceptor diameters and disc structure distances for Wt mouse rod cells agree well with previously published data, and even with those that used non-fixed, vitrified photoreceptor samples (34). FIB-SEM examination of photoreceptors from the Nrl⁻/⁻ mouse revealed structural changes that accompany this disease. Photoreceptor packing was severely disrupted, with Nrl⁻/⁻ photoreceptors more loosely packed (Figure 20C) than rods in Wt mouse retina. Furthermore, reconstructions of individual Nrl⁻/⁻ photoreceptors revealed that there was considerable disruption in the normal packing of internal photoreceptor discs, with abnormal structures collected at the head of photoreceptor outer segments at the RPE interface (Figure 20D). This abnormal accumulation indicates that the discs are not being properly removed from the photoreceptor, signaling a defect in the photoreceptor turnover and phagocytosis process. This led to investigation of larger portions of the retina to accurately capture this dynamic circadian process.

2.3.8 Evidence for aberrant phagocytosis in ESCS disease

Phagocytosis is a dynamic process that occurs across the entire retina. Therefore a hybrid SEM technique, serial block face (SBF) imaging, was used to image a large area of the retina and capture serial sections at sufficient resolution of the photoreceptor-RPE interface to identify any abnormalities that might relate to this process. The block face
was imaged by backscattered electrons after a 100 nm-section was removed by a microtome inside the SEM. This process was repeated to allow sectioning and imaging of the entire RPE to visualize the phagocytic process that occurs during OS disc shedding. SBF-SEM imaging revealed that the photoreceptor-RPE interface in the Wt sample has an orderly architecture with tightly stacked rods apposed to the interface where shed disc membranes are clearly engulfed (Figure 21A-B). Moreover, three-dimensional reconstructions revealed that these phagosomes were present throughout the RPE (Figure 21C), demonstrating that this technique can capture a dynamic process occurring across multiple planes of the retina. When the Nrl<sup>/−</sup> mouse retina was subjected to SBF-SEM imaging, the photoreceptors were not tightly packed at the RPE interface and they also exhibited the abnormal associations with the RPE previously noted. Furthermore, the aberrant diseased OS head phenotype was visible in some sections (Figure 21D-E), but no phagosomes were seen throughout the retina, despite sectioning through multiple RPE cells. Given that a proportion of cone photoreceptors is renewed daily (67, 144), it is noteworthy that not a single phagosome was identified, indicating severely reduced RPE phagocytosis of OS in this ESCS disease model. Three-dimensional reconstructions further illustrated the lack of phagosomes in the RPE and confirmed the presence of abnormal photoreceptor OS heads at the RPE interface (Figure 21F).

The compromised phagocytic process identified in Nrl<sup>/−</sup> mice was subsequently confirmed by staining for phagosomes at the RPE-photoreceptor interface. Cryosectioned retinas were stained with an antibody against phosphotidylserine (PS), the most abundant anionic phospholipid (198) and a key component of phagosomes (199, 200), asymmetrically situated in the inner leaflet of their plasma membranes (201). Because PS
is present in most cell membranes (202), low detergent concentrations (0.3% Triton) were used to selectively detect PS staining of shed disc membranes. In the Wt mouse retina, PS staining identified phagosomes at the photoreceptor-RPE interface (Figure 22). PS staining co-localized with shed discs labeled for cone opsin confirming that staining at the photoreceptor-RPE interface truly captured shed disc phagosomes (Figure 22A-D). Light and confocal microscopy of cryo-sectioned retinas and stained eye whole mounts also revealed that this phagosome staining was located at the RPE-photoreceptor plane interface (Figure 22E-F). When the same experiments were performed with Nrl⁻/⁻ mice, there was no such staining at the photoreceptor-RPE interface, either in cryo-sectioned retinas (Figure 22G) or eye whole mounts (Figure 22H). Similar experiments were done with an anti-annexin V antibody, which also recognizes PS albeit less specifically (203), with results consistent with those obtained with the anti-PS antibody. Because phagocytosis depends on both proper signaling from shed disc packets and recognition of this signal by the neighboring RPE, it was finally necessary to understand if the phagocytic defect observed in Nrl⁻/⁻ mice was due to an inherent abnormality of the photoreceptors or the RPE.

2.3.9 ESCS phenotype attributed to photoreceptor abnormalities rather than a RPE defect

The RPE plays a critical role in retinal maintenance. Thus the defect in Nrl⁻/⁻ mice and human ESCS patients may be attributable, not only to aberrant photoreceptors that result in this disease, but also to defective RPE cells that interface with these photoreceptors. To investigate these possibilities, isolated RPE cells from Wt and Nrl⁻/⁻ eyes were cultured (172). The yield from both type of mice was comparable. Purified OS
membrane vesicles from Wt and Nrl−/− animals then were used to test the phagocytotic activity of cultured RPE cells (174). The OS vesicles were not always intact when isolated, especially those from Nrl−/− animals due to interconnections of the cone-like discs, and thus would produce membranes with PS exposed to RPE cells that would promote normal phagocytosis. Signals were noted to emanate selectively from OS membrane vesicles that had been ingested by the RPE rather than any other source (Figure 23A-C). Wt RPE cells, when challenged with OS from Wt mice, phagocytosed these membranes as expected (Figure 23D-F). When the Wt RPE cells were challenged with OS from Nrl−/− mice, the RPE phagocytosed these membranes as well (Figure 23G-I). Moreover, RPE cells cultured from Nrl−/− mice also phagocytosed OS from Wt mice (Figure 23J-L) as well as from Nrl−/− animals (Figure 23M-O). These results indicated that the Nrl−/− defect was not in the RPE, but rather it was related to photoreceptors and their interface with the RPE.

2.4 Discussion and conclusions

The details of abnormal photoreceptor development resulting from mutations in NR2E3 and NRL genes have captured the interest of developmental biologists for nearly two decades. However, an equally important but less explored feature of ESCS is the progressive retinal degeneration that leads to blindness in these patients (112, 113, 146, 204). Retinal degeneration in ESCS has been postulated to be secondary to a defective photoreceptor maintenance function of NR2E3 or a combination of cell proliferation and death (113, 151, 177), but specifics have been lacking. To understand this unique human condition, an appropriate animal model is required. Knockout of the Nrl transcription
factor in mice produces a retina that is overpopulated with S-cone-like photoreceptors along with a complete absence of normal rod photoreceptors. Morphological assessment of this Nrl\(^{-}\) mouse model revealed that the postnatal perturbation of retinal organization (53, 108) was similar to the disorganized retinal layering noted in post-mortem donor retinas of ESCS patients (113, 151). In this study, by comparing and following ESCS patients, by in vivo and ex vivo imaging it was shown that the Nrl\(^{-}\) mouse model approximates the phenotypic features of human ESCS. Thus the Nrl\(^{-}\) mouse allowed for the probing of the molecular mechanisms of ESCS-induced retinal degeneration.

Comprehensive analysis of the murine eye and retina transcriptomes of Wt and Nrl\(^{-}\) mice by RNA-Seq allowed greater understanding of the global transcriptional mis-regulation that results in aberrant, unstable photoreceptors of the Nrl\(^{-}\) mouse retina. Interestingly, the RNA-Seq analysis reveals that many signaling networks, such as Notch and Hedgehog, needed for normal photoreceptor maintenance and retinal lamination are mis-regulated even in adult Nrl\(^{-}\) mice. Proper retinal cell type specification is heavily reliant on both Notch (152, 205) and Hedgehog signaling (189, 190), because depletion or pharmacological inhibition of Notch1 or Dhh in the retina causes progenitor cells to differentiate prematurely. Notably, Notch1 inhibition causes commitment of retinal progenitor cells to a cone photoreceptor fate, preferentially specifying S-cones (152, 153, 206). Furthermore, both Notch1 and Dhh are critical for proper retinal morphology, as their depletion in the retina causes rosette formation in the ONL (180, 207, 208). Precocious S-cone formation and ONL rosette formation resulting from aberrations in Notch and Hedgehog in the retina are consistent with what is observed in the mature Nrl\(^{-}\) retina.
The mis-regulation of Hedgehog and Notch pathways is evident by almost complete absence of Dhh in Nrl\(^{-/-}\) retina compared to Wt and up-regulation of bHLH factors in Nrl\(^{+/+}\) retina. The latter are normally suppressed by proper Notch signaling. Examples include neurogenic differentiation 1 (NeuroD1) and Atoh7, genes responsible for committing cells to the earliest cell fates of S-cone photoreceptors and ganglion cells, respectively. NeuroD1 alone cannot commit cells to the S-cone fate, but can do so in cooperation with Six6, another transcript upregulated in the Nrl\(^{-/-}\) retina. Six6 can be activated by NeuroD1 (209). Furthermore, Eya1, in the pathway of canonical Pax signaling is another transcript highly elevated in the Nrl\(^{-/-}\) retina. Although elevated levels of NeuroD1 and Six6 can commit cells to a S-cone fate, a prolonged increase of Six6 expression leads to disruption of photoreceptor maturation, indicating a regulatory factor is needed to down-regulate Six6 activity after its induction (209). It was hypothesized that the regulatory factor that could control terminal photoreceptor differentiation in this pathway is sine oculis-related homeobox 6 homolog opposite strand transcript 1 (Six6os1), which is highly up-regulated in the Nrl\(^{-/-}\) eye and retina (Figure 24A). Opposite strand transcripts are natural antisense transcripts that can be involved in gene regulation, and recently, it was predicted that both Six6os1 and Crxos1 might encode putative protein products and thereby play a major role in photoreceptor development (210). Altogether, RNA-Seq based elucidation of the eye and retinal transcriptomes revealed transcriptional mis-regulation in the mature Nrl\(^{-/-}\) mouse. These factors along with others identified in this study can potentially play a crucial role in cone photoreceptor maintenance (Figure 24B). Indeed, proteins encoded by those unique
genes found from this study to have no annotated function could prove especially attractive candidates for this role.

Although the RNA-Seq study identified transcriptional mis-regulation that could affect normal cone photoreceptor maintenance, it also provided a potential molecular mechanism for ESCS induced retinal degeneration due to defective photoreceptor phagocytosis. RNA-Seq revealed down-regulation of genes involved in photoreceptor phagocytosis such as tubby (Tub) and tubby-like protein 1 (Tulp1) (195) and possibly a recently described PS flippase in photoreceptor disc membranes, Atp8a2 (211). This prompted a more detailed study of the photoreceptor-RPE interface with high resolution imaging methods. Compared to Wt rods correctly apposed at the RPE interface, ESCS photoreceptors examined by TEM demonstrated abnormal interactions with the RPE. Phagocytotic material shed from OS could not be detected across the retinas of multiple Nrl–/– animals. Instead, the OS layer displayed a build-up of vacuole-like material in the heads of the photoreceptor, likely accounting for the bulbous OS head structures identified by SEM imaging. This phenotype progressed with increasing age such that the OS became devoid of discs. Thin sections also supported this view. However, such data do not illustrate the dynamic process of phagocytosis across the retina. Phagosomes could be lacking in any given section because only ~10% of photoreceptors may be shedding at any one time. Therefore SBF-SEM was utilized to section through an entire RPE cell in contact with hundreds of photoreceptor cells. This strategy allowed all phagocytotic events to be identified in Wt mouse retina at the morning peak of phagocytosis. In contrast, when the Nrl–/– retina was studied using the same approach, no phagosomes were detected. This apparent defect in phagocytosis was then validated biochemically. Retinas
from Wt mice stained with phagosomal markers such as PS and annexin V exhibited phagosomes at the photoreceptor-RPE interface. There was a complete absence of such staining in Nrl<sup>−/−</sup> mouse retina. Thus, both the defect in phagocytosis and the degenerative component of ESCS seem attributable to aberrant photoreceptors in the retina rather than a combination of photoreceptor and RPE cell dysfunction. This was consistent with the RNA-Seq study where the critical receptor tyrosine kinase involved in phagocytosis, *Mertk*, was unchanged at the transcriptional level between Wt and Nrl<sup>−/−</sup> mice. The aberrant photoreceptor hypothesis was further supported by challenging cultured Wt and Nrl<sup>−/−</sup>RPE cells with outer segment membranes. In the challenge assay, the fed outer segments are not intact and thus broken pieces presenting PS will readily be phagocytosed by the RPE cells. Both Wt and Nrl<sup>−/−</sup>RPE cells revealed comparable phagocytotic activity, indicating that the defect in phagocytosis was independent of an impairment in RPE function. Instead, the aberrant packing and spacing of the photoreceptors in ESCS disrupts the normal phagocytosis machinery of shed photoreceptor discs.

Based on this phagocytotic defect found in Nrl<sup>−/−</sup> mice, it was hypothesized that it is the precipitating cause of the retinal degeneration that occurs in human and murine ESCS (*Figure 24C*). Previous investigators have speculated that retinal degeneration in ESCS is secondary to a postnatal photoreceptor maintenance function of *NR2E3*, for example (113, 212). However, the dysplasia in human ESCS and murine models, evidenced by rosettes (or whorls), is not unique to ESCS but is a feature of many retinal pathological processes (213). Patchy loss of laminar integrity and abnormal photoreceptor-RPE interactions are dramatic features accompanying the rosettes in many
diseases, but there is no direct evidence that this causes progressive retinal degeneration. Even the hypothesis that overcrowding due to retinal folding or rosette formations that may be relieved by photoreceptor degeneration has been disputed (213). Phagocytosis of shed disc packets from the OS of photoreceptors is essential for normal function of these cells (143). Without this process, there is a build-up of material that becomes toxic to the cell over time as demonstrated in The Royal College of Surgeons rat, which is defective for *Mertk* (214, 215). Similarly, impairment of phagocytosis in the *Nrl*<sup>−/−</sup> retina could progressively cause a build-up of toxic materials that leads to degeneration. This build-up of toxic compounds would produce a fluorescent signal in the retina, much like the one we identified in ESCS patients (Figure 11E).

In summary, it has been shown that photoreceptors in the *Nrl*<sup>−/−</sup> retina have robust expression of S-cone opsin and they display an aberrant packing and morphology leading to progressive degeneration attributable to a defect in normal photoreceptor phagocytosis. Changes in the transcriptional landscape of the *Nrl*<sup>−/−</sup> eye result in the expression of a unique sub-set of photoreceptors genes at levels that differ from those of native rods or cones. The developmental defect that affects the photoreceptor cell fate also appears to have a detrimental effect on the normal retinal microenvironment. Thus, the inherent defect in phagocytosis in the *Nrl*<sup>−/−</sup> retina observed in this work is likely caused by changes in the normal transcriptional landscape that causes an overpopulation of ESCS photoreceptors in the retina. In animals, such as the tree shrew, that possess retinas that are almost completely populated with cones, phagocytosis occurs normally (144). The produced mutant photoreceptor cells in ESCS have a lower density than photoreceptor cells in Wt rod or cone-dominated retina as well as disruption in expression of key
homeostatic genes, including genes involved in proper photoreceptor phagocytosis and maintenance. This could account for their instability. These changes make ESCS photoreceptors unstable, producing retinal degeneration at an early age.
Table 1. GO term breakdown of transcript reads across different RNA-Seq experiments with Wt and \textit{Nrl}^{−/−} tissues\textsuperscript{1}.

<table>
<thead>
<tr>
<th>Go Term</th>
<th>Wt Retina</th>
<th>\textit{Nrl}^{−/−} Retina</th>
<th>Wt Eye</th>
<th>\textit{Nrl}^{−/−} Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy</td>
<td>28 (0.24)</td>
<td>27 (0.23)</td>
<td>28 (0.21)</td>
<td>28 (0.21)</td>
</tr>
<tr>
<td>Binding activity</td>
<td>979 (8.38)</td>
<td>995 (8.45)</td>
<td>1186 (8.85)</td>
<td>1190 (8.90)</td>
</tr>
<tr>
<td>Biogenesis</td>
<td>92 (0.79)</td>
<td>94 (0.80)</td>
<td>102 (0.76)</td>
<td>103 (0.77)</td>
</tr>
<tr>
<td>Catalytic activity</td>
<td>167 (1.43)</td>
<td>172 (1.46)</td>
<td>189 (1.41)</td>
<td>192 (1.44)</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>265 (2.27)</td>
<td>257 (2.18)</td>
<td>330 (2.46)</td>
<td>329 (2.46)</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>292 (2.50)</td>
<td>296 (2.51)</td>
<td>353 (2.63)</td>
<td>349 (2.61)</td>
</tr>
<tr>
<td>Cell death</td>
<td>165 (1.41)</td>
<td>173 (1.47)</td>
<td>203 (1.51)</td>
<td>202 (1.51)</td>
</tr>
<tr>
<td>Cytoskeleton organization</td>
<td>158 (1.35)</td>
<td>161 (1.37)</td>
<td>183 (1.37)</td>
<td>181 (1.35)</td>
</tr>
<tr>
<td>Developmental process</td>
<td>159 (1.36)</td>
<td>163 (1.38)</td>
<td>203 (1.51)</td>
<td>201 (1.50)</td>
</tr>
<tr>
<td>DNA repair</td>
<td>138 (1.18)</td>
<td>140 (1.19)</td>
<td>159 (1.19)</td>
<td>157 (1.17)</td>
</tr>
<tr>
<td>Homeostatic process</td>
<td>112 (0.96)</td>
<td>113 (0.96)</td>
<td>123 (0.92)</td>
<td>122 (0.91)</td>
</tr>
<tr>
<td>No term</td>
<td>2314 (19.82)</td>
<td>2341 (19.88)</td>
<td>2570 (19.17)</td>
<td>2599 (19.44)</td>
</tr>
<tr>
<td>Protein folding</td>
<td>98 (0.84)</td>
<td>98 (0.83)</td>
<td>101 (0.75)</td>
<td>102 (0.76)</td>
</tr>
<tr>
<td>Protein modification</td>
<td>421 (3.61)</td>
<td>418 (3.55)</td>
<td>508 (3.79)</td>
<td>500 (3.74)</td>
</tr>
<tr>
<td>Regulatory process</td>
<td>228 (1.95)</td>
<td>231 (1.96)</td>
<td>271 (2.02)</td>
<td>271 (2.03)</td>
</tr>
<tr>
<td>RNA processing</td>
<td>362 (3.10)</td>
<td>360 (3.06)</td>
<td>392 (2.92)</td>
<td>391 (2.92)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>1126 (9.64)</td>
<td>1131 (9.60)</td>
<td>1354 (10.10)</td>
<td>1337 (10.00)</td>
</tr>
<tr>
<td>Structural molecule activity</td>
<td>79 (0.68)</td>
<td>83 (0.70)</td>
<td>107 (0.80)</td>
<td>107 (0.80)</td>
</tr>
<tr>
<td>System process</td>
<td>55 (0.47)</td>
<td>61 (0.52)</td>
<td>86 (0.64)</td>
<td>91 (0.68)</td>
</tr>
<tr>
<td>Transcription</td>
<td>1164 (9.97)</td>
<td>1166 (9.90)</td>
<td>1255 (9.36)</td>
<td>1250 (9.35)</td>
</tr>
<tr>
<td>Translation</td>
<td>284 (2.43)</td>
<td>283 (2.40)</td>
<td>286 (2.13)</td>
<td>286 (2.14)</td>
</tr>
<tr>
<td>Transport</td>
<td>1287 (11.02)</td>
<td>1286 (10.92)</td>
<td>1434 (10.70)</td>
<td>1404 (10.50)</td>
</tr>
<tr>
<td>Total transcripts ((≥1))</td>
<td>11677</td>
<td>11778</td>
<td>13406</td>
<td>13368</td>
</tr>
</tbody>
</table>

\textsuperscript{1} All transcripts detected at a level of 1 FPKM in Wt and \textit{Nrl}^{−/−} tissue samples were categorized by GoTerm categorization using Amigo v1.8. There were less total transcripts in the retina sample runs since compared to the eye since we were examining a more specialized tissue of the whole. The number of transcripts in each category with percentages of total number of transcripts for that category shown in parenthesis did not greatly vary between Wt and \textit{Nrl}^{−/−} samples. This indicated that the knockout of \textit{Nrl} causes relatively subtle differences in the overall gene network to mimic the human ESCS phenotype.
Table 2. Fold changes of selected transcripts in Nrf^−/− relative to Wt tissue across different experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>RT-PCR</th>
<th>Retina RNA-Seq</th>
<th>Eye RNA-Seq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abca4</td>
<td>-2.94</td>
<td>-3.05</td>
<td>-3.98</td>
</tr>
<tr>
<td>Atp8a2</td>
<td>-2.22</td>
<td>-3.02</td>
<td>-3.9</td>
</tr>
<tr>
<td>Atoh7</td>
<td>11.44</td>
<td>6.07</td>
<td>4.83</td>
</tr>
<tr>
<td>Bmp15</td>
<td>3.48</td>
<td>8.75</td>
<td>4.96</td>
</tr>
<tr>
<td>Crx</td>
<td>-1.22</td>
<td>-1.02</td>
<td>-1.02</td>
</tr>
<tr>
<td>Egr1</td>
<td>18.26</td>
<td>7.46</td>
<td>11.20</td>
</tr>
<tr>
<td>Eya1</td>
<td>37.48</td>
<td>24.35</td>
<td>5.26</td>
</tr>
<tr>
<td>Gdf11</td>
<td>-1.85</td>
<td>-3.45</td>
<td>-2.50</td>
</tr>
<tr>
<td>Neurod1</td>
<td>2.57</td>
<td>2.20</td>
<td>2.80</td>
</tr>
<tr>
<td>Notch1</td>
<td>1.08</td>
<td>1.21</td>
<td>0.62</td>
</tr>
<tr>
<td>Opn1sw</td>
<td>39.32</td>
<td>25.89</td>
<td>22.93</td>
</tr>
<tr>
<td>Otx2</td>
<td>1.73</td>
<td>1.66</td>
<td>1.39</td>
</tr>
<tr>
<td>Prdm1</td>
<td>4.74</td>
<td>5.33</td>
<td>2.93</td>
</tr>
<tr>
<td>Rxrg</td>
<td>2.81</td>
<td>5.96</td>
<td>3.52</td>
</tr>
<tr>
<td>Six6</td>
<td>2.75</td>
<td>5.26</td>
<td>4.87</td>
</tr>
<tr>
<td>Six6os1</td>
<td>7.89</td>
<td>25.83</td>
<td>37.91</td>
</tr>
<tr>
<td>Thrb</td>
<td>1.24</td>
<td>1.27</td>
<td>1.37</td>
</tr>
<tr>
<td>Wnt9b</td>
<td>-4.55</td>
<td>-5.88</td>
<td>-3.92</td>
</tr>
</tbody>
</table>

1The fold change of selected transcripts was well preserved across different experiments. The targets chosen for validation included genes whose expression changed greatly (Opn1sw, Eya1, Egr1), genes with more subtle fold changes (Gdf11, Otx2, Thrb) and Crx, which had no significant change. Fold changes determined by RNA-Seq correlated well with changes in tissue expression of transcripts in both eye and retina.
Table 3. Transcript levels (in FPKM) of visual cycle proteins in Nrl<sup>−/−</sup> relative to Wt tissue across different RNA-Seq runs<sup>1</sup>.

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT Retina RNA-Seq</th>
<th>Nrl&lt;sup&gt;−/−&lt;/sup&gt; Retina RNA-Seq</th>
<th>Fold Difference&lt;sup&gt;2&lt;/sup&gt;</th>
<th>WT Eye RNA-Seq</th>
<th>Nrl&lt;sup&gt;−/−&lt;/sup&gt; Eye RNA-Seq</th>
<th>Fold Difference&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abca4</td>
<td>175.71</td>
<td>57.62</td>
<td>0.33</td>
<td>90.25</td>
<td>22.51</td>
<td>0.25</td>
</tr>
<tr>
<td>Arr3</td>
<td>88.55</td>
<td>578.24</td>
<td>6.53</td>
<td>62.25</td>
<td>302</td>
<td>4.85</td>
</tr>
<tr>
<td>Cnga1</td>
<td>198.7</td>
<td>0.04</td>
<td>-2</td>
<td>148.55</td>
<td>0</td>
<td>-2</td>
</tr>
<tr>
<td>Cnga3</td>
<td>4.63</td>
<td>44.45</td>
<td>9.60</td>
<td>2.18</td>
<td>21.64</td>
<td>9.93</td>
</tr>
<tr>
<td>Cngb3</td>
<td>2.66</td>
<td>84.83</td>
<td>31.89</td>
<td>1.34</td>
<td>42.98</td>
<td>32.07</td>
</tr>
<tr>
<td>Gnat1</td>
<td>7281.64</td>
<td>1.53</td>
<td>-2</td>
<td>4311.21</td>
<td>1.02</td>
<td>-2</td>
</tr>
<tr>
<td>Gnat2</td>
<td>100.61</td>
<td>1924.72</td>
<td>19.13</td>
<td>67.4</td>
<td>951.07</td>
<td>14.11</td>
</tr>
<tr>
<td>Gnb1</td>
<td>1472.44</td>
<td>67.23</td>
<td>0.05</td>
<td>1400.74</td>
<td>121.52</td>
<td>0.09</td>
</tr>
<tr>
<td>Gnb2</td>
<td>76.18</td>
<td>88.58</td>
<td>1.16</td>
<td>124.9</td>
<td>92.93</td>
<td>0.74</td>
</tr>
<tr>
<td>Gnb3</td>
<td>214.35</td>
<td>1481.55</td>
<td>6.91</td>
<td>144.04</td>
<td>815.01</td>
<td>5.66</td>
</tr>
<tr>
<td>Gngt1</td>
<td>304.41</td>
<td>574.91</td>
<td>1.89</td>
<td>209.38</td>
<td>591.24</td>
<td>2.82</td>
</tr>
<tr>
<td>Gngt2</td>
<td>60.66</td>
<td>1379.01</td>
<td>22.73</td>
<td>91.18</td>
<td>1465.48</td>
<td>16.07</td>
</tr>
<tr>
<td>Guca1a</td>
<td>720.15</td>
<td>1150.14</td>
<td>1.60</td>
<td>749.33</td>
<td>553.63</td>
<td>0.74</td>
</tr>
<tr>
<td>Guca1b</td>
<td>827.36</td>
<td>64.27</td>
<td>0.08</td>
<td>625.54</td>
<td>25.57</td>
<td>0.04</td>
</tr>
<tr>
<td>Guca1a3</td>
<td>33.47</td>
<td>39.42</td>
<td>1.18</td>
<td>19.16</td>
<td>25.01</td>
<td>1.31</td>
</tr>
<tr>
<td>Guca1b3</td>
<td>32.67</td>
<td>39.5</td>
<td>1.21</td>
<td>38.38</td>
<td>56.48</td>
<td>1.47</td>
</tr>
<tr>
<td>Guce2e</td>
<td>114.93</td>
<td>22.17</td>
<td>0.19</td>
<td>48.5</td>
<td>15.56</td>
<td>0.32</td>
</tr>
<tr>
<td>Guce2f</td>
<td>20.73</td>
<td>0.25</td>
<td>0.01</td>
<td>10.82</td>
<td>0.37</td>
<td>0.03</td>
</tr>
<tr>
<td>Lrat</td>
<td>2.36</td>
<td>3.5</td>
<td>1.48</td>
<td>14.64</td>
<td>15.01</td>
<td>1.03</td>
</tr>
<tr>
<td>Opn1mw</td>
<td>137.65</td>
<td>246.42</td>
<td>1.79</td>
<td>49.12</td>
<td>136.56</td>
<td>2.78</td>
</tr>
<tr>
<td>Opn1sw</td>
<td>191.93</td>
<td>4968.88</td>
<td>25.89</td>
<td>120.49</td>
<td>2762.3</td>
<td>22.93</td>
</tr>
<tr>
<td>Pde6a</td>
<td>492.09</td>
<td>30.48</td>
<td>0.06</td>
<td>243.91</td>
<td>12.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Pde6b</td>
<td>736.22</td>
<td>26.53</td>
<td>0.04</td>
<td>527.87</td>
<td>16.87</td>
<td>0.03</td>
</tr>
<tr>
<td>Pde6c</td>
<td>30.13</td>
<td>564.61</td>
<td>18.74</td>
<td>31.17</td>
<td>512.77</td>
<td>16.45</td>
</tr>
<tr>
<td>Pde6d</td>
<td>68.19</td>
<td>118.06</td>
<td>1.73</td>
<td>54.08</td>
<td>85.73</td>
<td>1.59</td>
</tr>
<tr>
<td>Pde6g</td>
<td>2250.16</td>
<td>1151.56</td>
<td>0.51</td>
<td>1561.58</td>
<td>639.69</td>
<td>0.41</td>
</tr>
<tr>
<td>Pde6h</td>
<td>166.22</td>
<td>1700.2</td>
<td>10.23</td>
<td>105.58</td>
<td>959.4</td>
<td>9.09</td>
</tr>
<tr>
<td>Ppp2r4</td>
<td>55.13</td>
<td>42.87</td>
<td>0.78</td>
<td>52.08</td>
<td>35.72</td>
<td>0.69</td>
</tr>
<tr>
<td>Rbp3</td>
<td>1093.27</td>
<td>1017.07</td>
<td>0.93</td>
<td>440.05</td>
<td>430.68</td>
<td>0.98</td>
</tr>
<tr>
<td>Rcvrn</td>
<td>1068.31</td>
<td>446.34</td>
<td>0.42</td>
<td>787.61</td>
<td>295.54</td>
<td>0.38</td>
</tr>
<tr>
<td>Rdh12</td>
<td>215.6</td>
<td>70.46</td>
<td>0.33</td>
<td>132.14</td>
<td>44.55</td>
<td>0.34</td>
</tr>
</tbody>
</table>
The differential expression pattern in the Nrl$^{-/-}$ mouse compared to Wt in transcript levels (FPKM) elucidates those transcripts that are essential for rod and cone function and maintenance. The fold differences, preserved across eye and retina RNA-Seq runs, illustrate noticeable enrichment of Cnga3, Cngb3, Gnb3, Gngt2, Pde6c, and Pde6h in the Nrl$^{-/-}$ mouse, highlighting their role in cone-like photoreceptor function and maintenance. Conversely, the noticeable reduction of Cnga1, Gnat1, Gnb1, Guca1b, Gucy2f, Pde6a, and Pde6b in the Nrl$^{-/-}$ mouse points out the importance of these transcripts in rod photoreceptor function and maintenance. In addition, many visual cycle proteins involved in retinoid metabolism such as Abca4, Rdh12, and Rdh5 have attenuated transcript expression in the Nrl$^{-/-}$ mouse, suggesting a mis-regulation of this process compared to the Wt mouse.

Fold differences cannot be accurately determined for transcripts that are almost completely absent in Nrl$^{-/-}$. 
Table 4. Transcript levels (in FPKM) of putative phagocytosis proteins in Nrl\(^{-/-}\) relative to Wt tissue across different RNA-Seq runs\(^1\).

<table>
<thead>
<tr>
<th>Gene</th>
<th>WT Retina RNA-Seq</th>
<th>Nrl(^{-/-}) Retina RNA-Seq</th>
<th>Fold Difference(^2)</th>
<th>WT Eye RNA-Seq</th>
<th>Nrl(^{-/-}) Eye RNA-Seq</th>
<th>Fold Difference(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anxa2</td>
<td>12.02</td>
<td>17.64</td>
<td>1.47</td>
<td>491</td>
<td>285.3</td>
<td>0.58</td>
</tr>
<tr>
<td>Axl</td>
<td>4.25</td>
<td>4.85</td>
<td>1.14</td>
<td>16.08</td>
<td>13.42</td>
<td>0.83</td>
</tr>
<tr>
<td>Cd36</td>
<td>0.45</td>
<td>1.13</td>
<td>2.51</td>
<td>11.18</td>
<td>30.48</td>
<td>2.73</td>
</tr>
<tr>
<td>Cd81</td>
<td>131.05</td>
<td>138.36</td>
<td>1.06</td>
<td>121.55</td>
<td>166.18</td>
<td>1.37</td>
</tr>
<tr>
<td>Gas6</td>
<td>125.26</td>
<td>78.05</td>
<td>0.62</td>
<td>175.97</td>
<td>95.2</td>
<td>0.54</td>
</tr>
<tr>
<td>Itgav</td>
<td>5.99</td>
<td>11.85</td>
<td>1.98</td>
<td>9.53</td>
<td>14.55</td>
<td>1.53</td>
</tr>
<tr>
<td>Itgb5</td>
<td>21.75</td>
<td>20.38</td>
<td>0.94</td>
<td>91.7</td>
<td>56.22</td>
<td>0.61</td>
</tr>
<tr>
<td>Mertk</td>
<td>1.82</td>
<td>2.44</td>
<td>1.34</td>
<td>3.38</td>
<td>2.97</td>
<td>0.88</td>
</tr>
<tr>
<td>Mfge8</td>
<td>190</td>
<td>155.59</td>
<td>0.82</td>
<td>253.06</td>
<td>173.92</td>
<td>0.69</td>
</tr>
<tr>
<td>Ptk2 (Fak)</td>
<td>9.37</td>
<td>12.39</td>
<td>1.32</td>
<td>15.04</td>
<td>14.8</td>
<td>0.98</td>
</tr>
<tr>
<td>Src</td>
<td>16.61</td>
<td>9.39</td>
<td>0.57</td>
<td>40.92</td>
<td>18.87</td>
<td>0.46</td>
</tr>
<tr>
<td>Tub</td>
<td>75.26</td>
<td>30.46</td>
<td>0.40</td>
<td>50.16</td>
<td>17.99</td>
<td>0.36</td>
</tr>
<tr>
<td>Tulp1</td>
<td>478.55</td>
<td>325.02</td>
<td>0.68</td>
<td>295.93</td>
<td>180.83</td>
<td>0.61</td>
</tr>
<tr>
<td>Tyro3</td>
<td>8.77</td>
<td>1.93</td>
<td>0.22</td>
<td>13.4</td>
<td>4.35</td>
<td>0.32</td>
</tr>
</tbody>
</table>

\(^1\)The differential expression pattern in the Nrl\(^{-/-}\) mouse compared to Wt in transcript levels (FPKM) elucidates those transcripts that are essential for rod and cone function and maintenance. The fold differences, preserved across eye and retina RNA-Seq runs, illustrate noticeable enrichment of Cnga3, Cngb3, Gnb3, Gngt2, Pde6c, and Pde6h in the Nrl\(^{-/-}\) mouse, highlighting their role in cone-like photoreceptor function and maintenance. Conversely, the noticeable reduction of Cnga1, Gnat1, Gnb1, Guca1b, Gucy2f, Pde6a, and Pde6b in the Nrl\(^{-/-}\) mouse points out the importance of these transcripts in rod photoreceptor function and maintenance. In addition, many visual cycle proteins involved in retinoid metabolism such as Abca4, Rdh12, and Rdh5 have attenuated transcript expression in the Nrl\(^{-/-}\) mouse, suggesting a mis-regulation of this process compared to the Wt mouse.

\(^2\)Fold differences cannot be accurately determined for transcripts that are almost completely absent in Nrl\(^{-/-}\).
Figure 11. Key features of human ESCS disease and the Nrl⁻/⁻ mouse model. (A) Topographic maps of visual sensitivity for S-cones (left column) and L/M-cones (middle column) in a normal subject and ESCS patients at different ages. Normally, S-cone sensitivities are less than those of L/M cones. In the ESCS patients, S-cone sensitivities are greater than L/M-cone sensitivities at the same loci. P1, a 13-year-old patient, has supernormal S-cone sensitivities; in P2 at two ages (40 and 48 years), S-cone sensitivities are normal or subnormal but still greater than those of co-localized L/M-cones and in the 8-year interval there is progressive loss of vision. Loci showing this positive (enhanced) difference are marked (+, right column). (B) Kinetic visual field extent for a large bright target (V-4e) as a function of age in 9 ESCS patients with longitudinal measurements spanning at least a decade. A decline with age seen in a proportion of these patients is attributable to progressive retinal degeneration. Inset: kinetic fields at two ages in ESCS P3 illustrates loss of field extent over a 19-year interval. (C) Cross-sectional optical coherence tomography (OCT) scans of retinal architecture along >10 mm of the horizontal meridian through the fovea (F) of ESCS P1 (below) compared with a normal subject (above). ONL (outer nuclear layer) and IS (inner segment) thicknesses are labeled (brackets at right of scans). Rectangles show temporal retinal region quantified in next panel. (D) Longitudinal reflectivity profiles (LRP) of outer retinal lamina in two representative normal subjects (left) and three ESCS patients (middle). Identifiable layers are labeled and colored. Among notable LRP features are thicker ONL and IS layers in these ESCS patients and abnormal structures between the IS and RPE. Quantification of IS thickness in 6 ESCS patients versus normal subjects (right) showed a significant difference. (E) En face autofluorescence images of the central fundus of two ESCS patients illustrating hyperautofluorescent features (white dots). Cross-sectional images in co-localized regions show dysmorphology with intraretinal hyper-reflective lesions (insets). (F) OCT of Wt and Nrl⁻/⁻ mouse retinas illustrates phenotypic changes in the Nrl⁻/⁻ retina resembling those in human ESCS disease, e.g. a hyper-reflective RPE-photoreceptor interface and nuclear layer rosette formation (indicated by *). ONL thicknesses are labeled (brackets at right of scans). (G) Three-dimensional spectral domain-OCT of Wt mice and Nrl⁻/⁻ mice indicates retinal disorganization caused by
rosette formation as well as an abnormal photoreceptor-RPE interaction in the Nrl<sup>−/−</sup> retina.
Figure 12. ESCS photoreceptors of $Nrl^{-/-}$ mice display aberrant packing. (A) Plastic block sectioning of $Wt$ mouse retina (n=5) shows ordered packing of photoreceptors and the ONL. (B-D) Cryo-sectioned $Wt$ eyes (n=10) and subsequent immunohistochemical localization highlight the laminar organization of $Wt$ cones and reveal that the entire cone photoreceptor sheath (peanut agglutinin stained green in panel B) and OS (S-cone opsin stained red in panel C) overlap perfectly (panel D). (E) Plastic block sectioning of $Nrl^{-/-}$ mouse retina (n=5) highlights disrupted retinal layers with a disjointed ONL and short disordered OS. (F-H) Cryo-sectioned $Nrl^{-/-}$ eyes (n=10) and subsequent immunohistochemical localization indicate disrupted OS architecture where the cone photoreceptor sheath (PNA stained green in panel F) and the OS (S-cone opsin stained red in panel G) do not always overlap (panel H). Scale bars in panels A-H are 5 μm.
Figure 13. Reproducibility of murine eye RNA-Seq experiments and globally differentially expressed genes detected between Wt and Nrt1/− whole eyes. (A) Comparison of two Wt murine eye technical replicate RNA-Seq experiments revealed an excellent correlation between independent runs (R²=0.98). Transcript levels were quantified as the number of sequence fragments per kilobase of exon model per million mapped reads (FPKM) (133). By using FPKM, we were able to normalize experimental results across different samples and runs to facilitate transcriptome comparison between Wt and Nrt1/− samples. Transcripts down to 1 FPKM were designated as expressed and used to generate the differential expression plot. Such transcripts also were annotated using AmiGO version 1.8 gene ontology software (216). More than 300 million mapped single reads were generated for all RNA-Seq analysis. This allowed the depth of sequencing to correlate FPKM accurately to quantities of reference transcripts in the tissues (133). Murine cDNA libraries were generated from pooled samples (217) of isolated mRNA of adult (4-week-old) whole eyes of 5 female Wt and 5 female Nrt1/− mice with a C57BL/J6 genetic background. Unique reads were mapped back to the known mouse genome (165). (B) A comparison of Wt and Nrt1/− whole eye runs is plotted to show the variation in transcript reads. The plots of Log FPKM of the eye runs of Wt and Nrt1/− illustrate that, whereas the majority of reads fall along the line representing equal expression, there are many transcripts that fall either well above or below the line that are differentially expressed. The highest expressed transcripts in both runs (circled) belong to the crystallin family of molecules found in the lens of the eye. Cnga1, Esrrb, Gnat1, Kcnj14, Nr2e3, Nrl, Rho, Slc24a1, and Susd3 (indicated by arrows) are among the highest expressed transcripts in the Wt retina whereas Gnat2, Gnb3, Gngt2, Opn1sw, Pde6c, and Pde6h (indicated by arrows) are among the highest expressed transcripts in the Nrt1/− retina RNA-Seq run. (C) The Wt eye RNA-Seq run, distinguished by a robust Nrl transcript presence, detected 13,406 transcripts at 1 FPKM or higher. (D) The assignment of transcripts to various GO term categories is shown as a pie-chart with the number of transcripts in each category listed. A large proportion of transcripts detected at ≥1 FPKM had no annotated function. (E) The resolution of the RNA-Seq run of the Nrt1/− eye revealed ablation of the Nrl transcript. The Nrt1/− construct was created by replacing coding exons 2 and 3 with a neomycin cassette (53), so the few reads that we did pick up
mapped upstream of exon 3, outside the area of targeted deletion. (F) The run detected 13,368 transcripts at 1 FPKM or higher and the assignment of transcripts to various GO term categories is shown as a pie-chart with the number of transcript in each category listed. Again, a large proportion of all transcripts detected at ≥ 1 FPKM had no annotated function.
Figure 14. RNA-Seq of Wt and Nrl+/− retinas reveals new differentially expressed genes arising from transcriptional mis-regulation. (A) The Wt retina RNA-Seq run, represented by robust Nrl transcript detection, detected 11,677 transcripts at 1 FPKM or higher. (B) The breakdown of GO term categories to which the transcripts are assigned is shown as a pie-chart with the number of transcript in each category indicated. (C) Single base resolution of the RNA-Seq run of Nrl+/− retina reveals ablation of Nrl transcript detection in the regions of exon 2 and 3 where there is a neomycin cassette. (D) The run detected 11,778 transcripts at 1 FPKM or higher and the breakdown of GO term categories to which the transcripts are assigned is shown as a pie-chart with the number of transcripts in each category indicated. (E) RNA-Seq runs of Wt and Nrl+/− retina are plotted to show their differential expression pattern. Plots of Log FPKM of the retinal runs of Wt and Nrl+/− illustrate that whereas the majority of reads fall along the line representing equal expression, there is a range of transcripts that fall either above or below the line that represent differentially expressed transcripts. Cnga1, Esrrb, Gnat1, Kcnj14, Nr2e3, Nrl, Rho, Slc24a1, and Susd3 (indicated by arrows) are among the highest expressed transcripts in the Wt retina whereas Clca3, Cngb3, Fabp7, Gnat2, Gnb3, Gngt2, Opn1sw, Pde6c, Pde6h, and Six6os1 (indicated by arrows) are among the highest expressed transcripts in the Nrl+/− retina RNA-Seq run. (F) Real time-polymerase chain reaction (RT-PCR) validated differential expression patterns detected by RNA-Seq. To validate differences from RNA-Seq experiments, retinal tissue from Wt and Nrl+/− mice was used for RT-PCR using probes against well characterized targets from previous studies as well as newly identified targets from the current RNA-Seq study. The RT-PCR results validated the RNA-Seq differential expression pattern ranging from those genes that were highly differentially expressed (Egr1, Opn1sw) to those with more subtle differential expression (Gdf11, Otx2, Thrb) and even those without a significant fold change (Crx). Bars are colored blue (RT-PCR of retina), red (RNA-Seq of whole eye), and green (RNA-Seq of retina). The current RNA-Seq experiment, when compared to two previous microarray studies looking at differential expression between Wt and Nrl+/− retina reveals more comprehensive and quantitative data. (G) The RNA-Seq data reveals 3659 unique transcripts up-regulated in the Nrl+/− retina compared to previous data sets, indicating a considerable amount of newly differentially expressed transcripts compared to previous
findings. Moreover, the table shows that whereas microarray studies can assess gross changes well (5 fold or greater), the more subtle changes in differential expression are more robustly characterized using RNA-Seq. (H) RNA-Seq reveals 2230 unique transcripts down-regulated in the Nrl−/− retina compared to previous data sets. The table again highlights the greater coverage of differential expression at lower thresholds using RNA-Seq.
Figure 15. ESCS photoreceptors of $Nrt^{+}$ mice display aberrant packing and OS morphology caused by build-up of material in OS heads and aberrant photoreceptor phagocytosis. Retinal whole mount confocal microscopy displays the tight packing of Wt mouse retinal photoreceptors in three dimensional space. (A) Cone OS (S-cone opsin antibody) and (B) cone sheath (peanut agglutinin) signals overlap and illustrate the packing of cones in normal retina. Staining of (C) cone OS and (D) rod OS (rhodopsin C-terminal 1D4 antibody) reveals that the retina is fully occupied by photoreceptors, especially densely packed rods. (E) SEM imaging of critical point dried Wt retina further emphasizes that photoreceptors pack tightly in the retina. (F) Closer examination by SEM shows that rod photoreceptors display their characteristic cylindrical shape. In contrast, $Nrt^{-}$ retinas exhibited disrupted photoreceptor packing with clusters of densely populated cones separated by empty patches. The cone-like OS (G) and extracellular sheath (H) signals overlap, but also are absent from some retinal patches. Staining of cone OS (I) and rod OS (J) reveals only ESCS photoreceptors. (K) SEM imaging of critical point dried $Nrt^{-}$ retina shows disrupted packing of photoreceptors in the retina with a less dense population of photoreceptors than Wt. (L) Closer examination by SEM highlights abnormal OS morphology with enlarged head structures. Thin sectioned retinas from Wt and $Nrt^{+}$ mice were prepared for TEM imaging. (M) Thin sectioning of Wt retina reveals the internal structure of photoreceptors. (N) Discrete stacked discs are seen in rod photoreceptors. Because these samples were prepared at the peak of photoreceptor turnover, (O) TEM imaging captures the disc shedding process and RPE mediated phagocytosis (indicated by *). (P) Thin sectioning of $Nrt^{-}$ retina shows a distinctive OS disc arrangement that differs from Wt rods. (Q) Discs retain some interconnections (indicated by black arrow) as well as connections to the plasma membrane. (R) Closer examination of ESCS photoreceptors reveals that most photoreceptors have enlarged head structures owing to build-up of material at the photoreceptor-RPE interface (indicated by *), that would not occur with normal phagocytosis. Scale bar is 5 µm in panels A-D and G-J; 1 µm in panels E, F, K, L, O, and R; and 250 nm in panels M, N, P, and Q.
**Figure 16.** TEM confirms that older *Nrl<sup>−/−</sup>* mouse retinas display full course of impaired phagocytotic degeneration. In 4-week-old mice, the *Nrl<sup>−/−</sup>* retina does not evidence marked degeneration, but by 8 weeks of age, we and others have noted that such degeneration has rapidly progressed. Eight-week-old *Nrl<sup>−/−</sup>* mouse retinal samples were prepared by thin sectioning to demonstrate that the phenotype seen in 4-week-old animals is now greatly exaggerated. (A) There are fewer photoreceptors in the *Nrl<sup>−/−</sup>* retina, and (B) the remaining photoreceptors (zoomed in view from A) exhibit completely disrupted OS structures with discs replaced by a population of vacuoles, indicating a progressive accumulation of material by these cells. Scale bar in panels A and B is 1 μm.
Figure 17. Layers of neatly stacked rod photoreceptors in Wt mouse retina. (A) A view of the trench created at the beginning of an experiment designed to visualize the interiors of the rod photoreceptors. (B) An electron beam image showing the outlines of each rod photoreceptor and the internal disc elements. (C) The resulting three-dimensional reconstruction of rods showing how neatly and tightly these cells are stacked in mouse retina. Scale bars in panels A and B are 10 and 5 μm, respectively.
Figure 18. Internal architecture of Wt mouse rod photoreceptors. (A) A view of rod photoreceptors in a critical point-dried sample of Wt mouse retina and (B) subsequent deposition of platinum on top of the region of interest to protect its surface from milling artifacts during sectioning. (C) The created trench allows visualization of the internal architecture of rod photoreceptors with (D) a zoomed image of a single rod photoreceptor used for reconstruction shown. (E) The resulting three-dimensional reconstruction of the rod photoreceptor and internal disc elements. The rod cell outer segment membrane is outlined in a transparent gray color with the internal discs shown in blue and green colors. Scale bars in panels A, B, C and D are 5, 10, 10, and 1 μm, respectively.
Figure 19. Internal architecture of \( Nrl^{--} \) photoreceptors. Shown are (A) Photoreceptors from a critical point dried sample of \( Nrl^{+} \) mouse retina and (B) subsequent deposition of platinum on top of a region of interest to protect the surface from milling artifacts during sectioning. (C) A created trench allows visualization of the internal architecture of \( Nrl^{--} \) photoreceptors with (D) a zoomed image of a single \( Nrl^{--} \) photoreceptor used for reconstruction shown. (E) The resulting three-dimensional reconstruction of an \( Nrl^{--} \) photoreceptor and its disrupted internal disc elements. Towards the inner segment, the disc elements stack normally and retain some connection to the outer segment. The \( Nrl^{--} \) photoreceptor inner segment is shown in gray and the outer segment in transparent gray. Disc elements inside the outer segment are shown in blue and green with the disrupted elements in red. Scale bars in panels A, B, C and D are 10, 10, 10, and 1 \( \mu \)m, respectively.
**Figure 20.** Three-dimensional reconstructions from FIB-SEM data reveal packing architecture and internal elements of photoreceptors and the changes that accompany ESCS disease. (A) Reconstructed rod photoreceptors from Wt mouse retina with a diameter of 1.2 ± 0.1 μm are shown to align and pack tightly together. Reconstructions of photoreceptor internal elements reveal that (B) discs in Wt mouse rods stack orderly with an average disc diameter of 1.1 μm ± 0.3 μm (n=41 discs). (C) The photoreceptor packing density is greatly reduced in the Nrl−/− retina, mainly due to the aberrant shapes of the outer segments, which have a diameter of 1.0 μm ± 0.2 μm. Meanwhile, (D) The Nrf−/− photoreceptor reveals abnormal structures at the RPE interface with these elements ranging in size from 0.4–0.8 μm.
Figure 21. SBF-SEM allows visualization of impaired phagocytosis present in ESCS retinal degeneration. Because photoreceptor disc phagocytosis is a dynamic process that occurs throughout the retina, SBF-SEM imaging was used to collect precise serial sections and investigate phagocytosis of shed discs. (A) In Wt retina, the photoreceptor-RPE interface is clearly visible with tight packing of rods opposed to the RPE. (B) Moreover, phagosomes (indicated by *) ingested by the RPE are clearly visible. (C) Three-dimensional reconstructions of collected data with Reconstruct allow visualization of multiple phagosomes (colored red) including the one shown in panels A and B (indicated by *) throughout a RPE cell (colored gray with the nucleus in blue) and also reveal the tight packing of rods (green and blue) in a plane. (D) In Nrl<sup>-/-</sup> retina, the photoreceptor-RPE interface is visible, but photoreceptors (indicated by *) are not as densely packed against the RPE. (E) Enlarged OS head structures of ESCS photoreceptors are seen (indicated by *) with less electron-dense material at the tips, indicating loss of OS material in that area. (F) Resulting three-dimensional reconstruction illustrates these enlarged headed photoreceptors (indicated by *) and their interactions with the RPE (colored gray with the nucleus in blue). Of note is the absence of any visible phagosomes within the modeled Nrl<sup>-/-</sup> RPE. Scale bar in panels is 1 µm.
Figure 22. Absent phagosome staining at the photoreceptor-RPE interface confirms impaired phagocytosis in Nrl−/− mice. The absence of proper phagocytosis was confirmed by biochemical staining for phosphatidylserine (PS) found on phagosomes and its localization with shed cone disc packets. (A) In Wt retina, shed cone opsin discs in red (cone opsin antibody) co-localize with phagosomes stained for PS in green (indicated by arrow) at the photoreceptor-RPE interface. (Note that not all the PS staining indicates phagosomes as PS dye also stains the RPE cell nucleus). A zoomed in view (B) shows a cone opsin disc (red, indicated by arrow), (C) PS phagosome staining (green indicated by arrow), and (D) co-localization of the two stains (indicated by arrow). (E) In samples of Wt mouse retina examined at the peak of phagocytosis, there is staining of phagosomes (red) at the photoreceptor-RPE interface (indicated by white arrows). (F) Through optical sectioning of the collected data, confocal imaging of the retina-RPE interface reveals that the PS signal is present at the photoreceptor-RPE interface. A still image of the tangential plane of these collected data is shown with three corresponding slices of data on the right indicating the layers containing nuclear (blue), cone sheath (green), and phagosome (red) signals. In contrast (G), comparable Nrl−/− retinal samples fail to exhibit staining for PS at the photoreceptor-RPE interface. (H) Confocal imaging of the Nrl−/− retina-RPE interface shows that no detectable PS signal is evident at the photoreceptor-RPE interface. Note that it appears that there is some PS staining in the Nrl−/− retina but it is not located at the interface. A still image of the tangential plane of the collected data is shown with the three slices of data on the right indicating the layers containing nuclear (blue), ESCS photoreceptor (green), and phagosome (red) signals. Staining: cone opsin for cone OS; PNA for cone sheaths; DAPI or Topro 3 nuclear stain; phosphatidylserine for phagosomes. Scale bar in panels is 5 µm.
**Figure 23.** Wt and *Nrl*<sup>−/−</sup> mouse RPE phagocytose both Wt and *Nrl*<sup>−/−</sup> photoreceptor OS membranes. First, the phagocytosis assay was run with no OSs as a negative control. (A) Wt mouse RPE in culture not subjected to photoreceptor challenge were just washed with FITC-labeled dye (B) where only faint background fluorescence was detected. The overlay of the two images is shown in (C). Then, Wt RPE cells (D) were challenged with isolated Wt photoreceptor OS membranes (E). Phagocytosis of the OS membranes was seen by the overlapping images (F). Also, Wt RPE cells (G) challenged with isolated *Nrl*<sup>−/−</sup> photoreceptor OS membranes (H) phagocytosed the OS membranes as evident in the overlapping images (I). *Nrl*<sup>−/−</sup> RPE cells (J) challenged with isolated Wt photoreceptor OS membranes (K) phagocytosed the OS membranes as evident in the overlapping image (L). Similarly, *Nrl*<sup>−/−</sup> RPE (M) challenged with isolated *Nrl*<sup>−/−</sup> photoreceptor OS membranes (N) phagocytosed the OS membranes as evident in the overlapping images (O).
Figure 24. Transcriptional mis-regulation causes precocious development of cone-like cells in the Nrl\(^{-/-}\) retina, which are then maintained by transcriptional networks that alter key homeostatic processes. (A) Increased levels of Eye1 in the Nrl\(^{-/-}\) retina can activate the expression of Six6, which causes RPC proliferation. Commitment of these retinal cells to an early cell fate requires a premature cell cycle exit. This is mediated by altered levels of Notch and Hedgehog transcriptional networks that produce increased levels of NeuroD1 and decreased levels of Dhh in the Nrl\(^{-/-}\) retina. Six6 and NeuroD1 together with Six6os1 exercising a possible regulatory role on Six6, synergize to promote the S-cone fate rather than alternative early cell fates. (B) The maintenance of the cone-like cells in the mature retina can be attributed to a series of genes involved in transcriptional control of retinoid metabolism, transport, cell cycling, and signal transduction. Unique transcripts identified by RNA-Seq to be at least 5-fold differentially expressed in Nrl\(^{-/-}\) versus Wt mouse retina provide a resource for identifying maintenance factors required for cone cell survival and the alterations that accompany disease. The up and down arrows indicate transcripts that are up and down-regulated, respectively, in Nrl\(^{-/-}\) compared to Wt retina as determined by RNA-Seq. (C) Transcriptional mis-regulation causes changes in the expression of key homeostatic genes involved in phagocytosis leading to the pathological degeneration in ESCS. The most critical receptor tyrosine kinase, Mertk, involved in RPE phagocytosis is unchanged in the Nrl\(^{-/-}\) retina compared to Wt. However, key homeostatic genes involved in photoreceptor OS phagocytosis and toxic metabolic movement, such as Abca4, Atp8a2, Tub, Tulp1, are down-regulated in the Nrl\(^{-/-}\) retina compared to Wt, thus contributing to the defect in photoreceptor phagocytosis. The down arrows indicate transcripts that are down-regulated in the Nrl\(^{-/-}\) retina compared to Wt, whereas the up/down arrow indicates those transcripts that were unchanged in expression as determined by RNA-Seq.
CHAPTER 3: DIFFERENTIAL BACKGROUND GENETIC NETWORKS DRIVE MULTIGENIC AGE-RELATED RETINAL DEGENERATION

Portions of this chapter were previously published in:

3.1 The multi-genic etiology of age-related retinal degeneration (ARD)

3.1.1 Age-related pathology in the eye and the genetic contributions influencing disease

Compared to Mendelian diseases such as ESCS, researchers have struggled to link genotype to phenotype of more common, yet complex diseases arising from such things as increasing age (218). Age-related disorders arise from failure of tissue maintenance and repair pathways that are accelerated by certain inherited and acquired factors (219). Long-lived non-dividing cells, such as neurons, have markedly reduced tolerance to damage (220), thus exhibiting the most pronounced age-related changes. Neuronal cells in the retina are an especially attractive model system to study this phenomenon owing to their accessibility and well understood physiology. Rod and cone photoreceptors are retinal neuronal cells that initiate visual perception, a function requiring a competent neighboring retinal pigmented epithelium (RPE) for their normal operation (221). In post-mitotic cells such as these photoreceptor and RPE cells, cellular senescence can ensue when shed oxidized photoreceptor outer segments (POS) are inadequately phagocytosed and digested by the RPE. This results in accumulation of damaged proteins, formation of toxic metabolic by-products, inflammatory cell invasion and cell death. RPE cells are the most affected because, in addition to processing POS, they serve as the conduit between photoreceptors and the choroidal blood supply for metabolite exchange (222). Acquisition of senescence-associated pathology stimulates cells to secrete various factors that contribute to tissue dysfunction. Conversely, adequate clearance of such cells can delay the onset of age-related tissue pathology (223).
Aging laboratory experimental animals represent important models to study age–related pathology, especially neurodegenerative disorders, which have been shown to be affected by their genetic backgrounds (224, 225). The A/J inbred mouse model, for instance, undergoes age–related retinal degeneration (ARD) much more rapidly than its albino, BALB/c, and pigmented C57BL/6J (B6) counterparts, and presents a natural model to study age–related pathological changes in the retina. Although different factors, such as those involved in inflammation and homeostatic processing originating from the photoreceptor and RPE layers have been identified as possible markers of disease (226), the sets of genes that trigger progression to the chronic disease state are still poorly understood. This stems from the fact that most genetic studies to date have been carried out with affected individuals with considerable disease progression. Moreover, lack of methodology sufficiently powerful to reveal the complex interplay of genes that trigger and promote the progression of ARD has limited our understanding of disease pathogenesis. Studies to date have mostly relied on quantitative trait loci (QTL) (224) or chromosome substituted strain (CSS) panels (227), but these methods cannot detect gene expression changes and single nucleotide polymorphisms (SNPs) on the global scale needed to identify distant interacting genetic factors. Deep sequencing technology offers a powerful tool to understand how global gene expression variations associate with complex multi-genic diseases.

3.2 Rationale and methodology to determine multi-genic contributions driving ARD

3.2.1 Rationale for research approach
Despite significant scientific progress, understanding the genetic contributions to mechanisms of complex age-related diseases becomes an even more urgent priority as human life expectancy increases and the burden on public health rises. Post-mitotic cells, such as neuronal cells of the retina, are most susceptible to age-related degeneration and different inbred strains of mice exhibiting varying degrees of ARD offer the opportunity to identify genetic factors contributing to this blinding process in the human population. Phenotypic variability based on genetic background differences observed in mice provides models for this complex human condition. Although few genetic markers of disease in ARD have been identified from genome wide association studies, the lack of a global understanding of how these few genes give rise to disease is reflected by relative paucity of effective compounds for early prevention and treatment of ARD. The goal of this work was to pose a unified model of ARD onset and pathogenesis by using powerful genetic and morphological approaches to serially study mice from a young age before phenotypic changes to older age where there is pronounced loss of retinal function. Moreover, by using natural background mice with varying rates of ARD, the goal was to understand how subtle genetic differences, as in the human population, can predispose and drive age-related disease in the retina. Complementation of high-resolution cellular assessment of ARD with RNA deep sequencing technology would allow for the identification of more global salient signatures of disease onset and mechanistic factors that contribute to progressive features of this blinding condition.

3.2.2 Materials and methods

Animals. A/J, BALB/c and C57BL/6 (B6) inbred mice, and A/J CSS mice were obtained from The Jackson Laboratory. Long-Evans rats were purchased from Harlan.
Laboratories. Nile rats were obtained from the laboratory of Dr. Laura Smale (Michigan State University). Mice and rats were housed in the animal facility at the CWRU School of Medicine where they were maintained on a standard chow diet in a 12 h light (~10 lux)/12 h dark cycle.

**Histology and immunohistochemistry (IHC).** Histological and IHC procedures were carried out as previously described (228). IHC sections were viewed with a Zeiss LSM 510 inverted Laser Scan Confocal Microscope.

**Electroretinograms.** All ERG procedures were performed by previously published methods (228).

**Retinoid and A2E analyses.** All experimental procedures related to extraction, derivatization, and separation of retinoids from dissected mouse eyes were carried out as described previously (228). Quantification of A2E by HPLC was achieved by comparison with known concentrations of pure synthetic A2E standards (229).

**Ultra−high resolution spectral−domain optical coherence tomography (SD−OCT) and scanning laser ophthalmoscopy (SLO) imaging.** Both ultra−high resolution SD−OCT (Bioptigen) and SLO (HRAII, Heidelberg Engineering) were employed for *in vivo* imaging of mouse retinas as previously described (230).

**Library preparation and Illumina RNA-Seq runs.** Mouse eye tissue libraries were prepared as described in the preceding chapter. Pooled total RNA samples from 5 eyes were used for each whole eye library preparation. Three separate biological replicate libraries were made from mice with either A/J, BALB/c or B6 backgrounds. Each murine eye library was run on one lane of the Genome Analyzer IIx in the CWRU Genomics Core Facility using 36, 50, or 79 base pair single−end sequencing. The number of
mapped single reads was 19.9, 22.5, and 21.7 million from the three different A/J eye libraries, 41.2, 38.9, and 27.4 million from the three different BALB/c eye libraries, and 16.8, 17.7, and 38.2 million from the three different B6 eye libraries. Data were processed and aligned with the University of California–Santa Cruz mouse genome assembly and transcript annotation (mm9) and processed as previously described to calculate FPKM statistics by gene, transcript, and exon. The output for CASAVA was visualized with the GenomeStudio RNA Sequencing Module (Illumina), which allowed comparisons between samples. The processed and raw fastq files can be found online at the National Center for Biotechnology Information gene expression omnibus site with the series accession number GSE38359.

**Pathway generation and analysis.** RNA–Seq data were analyzed with Ingenuity pathway analysis software (Ingenuity Systems). Average FPKM values from RNA–Seq runs of A/J, BALB/c, and B6 mouse eyes were uploaded along with gene identifiers and statistical p-value calculations. Core analysis of a set of 11,339 mapped genes was done to identify perturbed molecular networks. Pathway generation of differentially expressed gene was also done by hand with Ingenuity, Pubmed database, and known retinal localization as guides.

**RT-PCR.** Two µg of isolated total retinal RNA from 3 pooled A/J, BALB/c, or B6 eye samples were converted to cDNA with the High Capacity RNA–to–cDNA kit (Applied Biosystems). All real–time experiments were done in triplicate with an ABI Step–One Plus qRT–PCR machine (Applied BioSystems). Fold changes were calculated based on differences in threshold cycles (C_t) between A/J or BALB/c and B6 samples after normalization to 18S rRNA.
Mouse eye cups were prepared as described in the preceding chapter. Blocks were cut with a microtome and ultra-thin sections (0.07 μm) were stained with uranyl acetate and adsorbed onto carbon grids. A Tecnai T12 EM (FEI) operating at 120 kV with a LaB₆ filament was used for final imaging.

**Two–photon microscopy.** Two–photon excitation microscopy was performed with a Leica TCS SP2 scanning head (Leica) attached to a DM IRBE2 inverted microscope stand with Leica LCS three–dimensional software. Laser pulses from a mode–locked Ti:Sapphire laser (Chameleon™–XR) were focused on the tissue sample by an HCX PL APO 40× oil immersion objective lens (NA = 1.25, Leica). Imaging and sample conditions were as described (167).

**In vivo detection of reactive oxygen species (ROS).** ROS detection *in vivo* was performed as previously published (231). The ROS probe, dihydroethidium (DHE, Invitrogen), at a dose of 20 mg/kg body weight was administered to mice via intraperitoneal injection 30 min prior to light exposure. Eye cups obtained after removing the cornea, lens and vitreous body from enucleated eye globes 3 h post light illumination were fixed in 4% paraformaldehyde. Cryosections were prepared from fixed eye cups and cut at 12 μm thickness for microscopic assessment of ROS signal fluorescence in the retina.

**Statistical analyses.** Experimental results were analyzed by the one way ANOVA–test with p values≤0.05 considered statistically significant. Data are presented graphically in figures as mean ± standard deviation.

**Study Approval.** All animal procedures and experiments were performed in accordance with U.S. animal protection laws and were approved by the CWRU Animal Care
Committees and conformed to both the recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

3.3 Results

3.3.1 A/J genetic background mice undergo pronounced age–related retinal degeneration

Retinal cross-section images revealed a prominent decrease in outer nuclear layer (ONL) thickness attributable to photoreceptor loss as well as pathological changes in the RPE layer of 8–month–old A/J mice as contrasted to 1–month–old animals (Figure 25A). Conversely, B6 mice displayed a negligible age–related decrease in ONL thickness and no other pathological changes (Figure 25A). Age–dependent ONL loss was observed globally in A/J mouse eyes (Figure 25B). Cone photoreceptor numbers also indicated a significant age–related decrease in A/J mice, whereas B6 mice exhibited only a slight decrease (Figure 25C). The decline in photoreceptor number in A/J mice compromised visual function as evidenced by attenuation of electroretinographic (ERG) responses (Figure 25D) and decreased levels of total retinoid content, especially the visual chromophore, 11–cis–retinal (Figure 26), at 8–months of age. The aged retinal phenotype was independent of light–induced or pigmentary contributions (224) as A/J mice reared in the dark also displayed a significant decline in ONL thickness at 8–months of age as compared to 8–month–old B6 mice (Figure 27) and 8–month–old albino BALB/c mice exhibited only a 20% decline in ONL thickness and had a similar cone population when compared to 8–month–old B6 mice (Figure 28). To better elucidate
factors that underlie the accelerated progression of this degenerative phenotype in A/J but not BALB/c or B6 mice, a more in-depth retinal examination of mice with different genetic backgrounds was undertaken.

### 3.3.2 ARD in A/J mice is accompanied by inflammatory cell infiltration and RPE cell pathology

*In vivo* fundus imaging with a scanning laser ophthalmoscope (SLO) revealed a notable increase in autofluorescent spots in the outer retinal layers of 8-month-old compared to 1-month-old A/J mice where the photoreceptors and RPE are located. No such age-related changes were observed in B6 mice (Figure 29A). Fundus autofluorescence levels did not correlate with amounts of age-related retinoid byproducts such as N-retinyl-N-retinylidene-ethanolamine (A2E) in the eyes of these mice (Figure 26A). These spots could, however, indicate immune cell activation and infiltration into the subretinal space (230). This was further supported by *in vivo* SD-OCT and plastic block sections both of which indicated possible infiltrative cells in 8-month-old A/J but not B6 mice (Figure 29B). Positive staining with the activated macrophage marker, Iba-1, indicated that some of the cells were of inflammatory origin whereas positive staining of other cells with the activated Müller cell marker, GFAP, indicated the stressed nature of the retinal environment of A/J but not B6 mice at 8-months of age (Figure 29C).

Age-dependent changes were further evaluated with high-resolution TEM imaging of horizontal cross-sections of the RPE–photoreceptor interface. As early as 3-months of age, when no notable ultrastructural or functional decline was evident
(Figure 25), A/J mice exhibited pathological changes in the RPE cell layer evidenced by swollen cells with abnormal intercellular inclusions resembling inadequately processed photoreceptor discs (Figure 30A). TEM imaging of retinal cross-sections showed abnormal processing of ingested photoreceptor discs by the RPE. These abnormalities were characterized by secondary pockets inside phagosomes (Figure 30B-C) and abnormal membrane formation that exposed the contents of the discs to the RPE cell cytoplasm (Figure 30D-E). In contrast, RPE cells imaged at 3–months of age in B6 mice revealed a smaller average cell width than A/J RPE cells with no pathological inclusions (Figure 30F). Furthermore, ingested discs appeared to be properly processed and transported in the B6 RPE cells (Figure 30G-J). Age-dependent pathology of RPE cells was also assessed by two-photon microscopy (TPM). There was a change in morphology and an increase in average RPE cell size in A/J mice from 1– to 8–months of age, whereas the size and morphology were well preserved with age in B6 mice (Figure 30K). The localization and function of a major RPE specific protein, RPE65 that is critical for visual chromophore regeneration (232), was investigated to assess if these pathologic changes translated into impairment of RPE cell function. Immunohistochemical (IHC) staining revealed that RPE65 was homogenously expressed in the RPE cell layer at 1–month of age, but then became heterogeneous in expression by 8–months of age in A/J mice (Figure 30L). This correlated with functional deterioration, as illustrated by a 75% depressed regeneration ratio of the visual chromophore, 11–cis–retinal, at 8–months of age in A/J mice (Figure 30M). This ratio was normal in A/J mice at 1–month of age (Figure 26C). No significant changes in this ratio or RPE65 localization were evident in B6 mice.
Although BALB/c mice displayed a mild retinal degenerative phenotype as compared to B6 mice, it was not nearly as pronounced as the phenotype seen in A/J mice. SLO imaging revealed an increase in autofluorescent spots at 8–months compared to 1–month of age in BALB/c mice (Figure 31A), but only mild immune cell infiltration was detected (Figure 31B) with no obvious pathological changes of the RPE cells (Figure 31C), suggesting that BALB/c mice possessed important retinal protective factors that curbed the inflammatory progression of ARD relative to A/J mice. These findings raised the question of what genetic differences between A/J, BALB/c and B6 mice could initiate the progression of RPE pathologic changes, leading to a chronic inflammatory state in A/J mice. To address this, genetic tools such as consomic strains and RNA deep sequencing technology were utilized to reveal multi–genic contributions that precipitate and perpetuate this disease.

### 3.3.3 RNA–Seq reveals differential genetic background contributions to the transcriptome

Twenty two CSSs of inbred mice, each carrying a single chromosome substituted from a donor strain (A/J) into a host background strain (B6) (Figure 32A) were used as a more powerful approach than QTL analysis to assess complex genetic traits (Figure 32). However, retinoid analysis (Figure 32B, D) as well as histological assessment (Figure 32C, E) of the CSS panel mice at 8–months of age revealed no significant phenotypic features of ARD. Moreover the lack of significant differences in CSSs 3 or 7 further demonstrated that neither lighting conditions (233) nor pigmentary status (234) were the major determinants to disease. Mis-localization of the centromeric portion of chromosome 1 (235) in the CSS panel was investigated but no genetic variation or
candidate genes from that region could account for the retinal phenotype observed. Thus, ARD in A/J mice is likely attributable to contributions from multiple chromosomes, necessitating a more global genetic approach.

To investigate the putative multi–genic etiology of ARD and identify more distant interacting genetic factors that contribute to its pathology, RNA–Seq of whole eye tissues from A/J, BALB/c, and B6 mice was carried out. To identify genetic factors that could trigger this pathology, 1–month–old mice from each genetic background were analyzed before obvious ARD–related phenotypic changes had occurred. Three biological replicates of ocular tissues from each strain were prepared for RNA–Seq to obtain adequate statistical power. Replicate results from each strain had high correlation coefficients when plotted against each other (Figure 33). A value of 1 fragment per kilobase per million reads (FPKM), equivalent to one transcript per cell (133), was used as the expression cutoff. Categorization of expressed transcripts, 12,606 in the A/J eye, 12,589 in the BALB/c eye, and 12,832 in the B6 eye, by GoTerm annotation did not reveal any significant differences between mouse strains at 1–month of age (Table 5).

Three biological replicate RNA–Seq runs provided an accurate determination of the differential expression profile between the three different mouse strains. RNA–Seq results were further corroborated with a cohort of RT–PCR probes (Figure 34A–B). The RNA–Seq data were further validated for visual cycle proteins by immunoblotting (Figure 34C). At least a 2–fold difference (p ≤ 0.05) was used as the cut–off to identify differentially expressed genes between mice with different genetic backgrounds. The RNA–Seq differential expression profile for A/J and B6 eyes revealed that whereas most genes overlapped in expression, there were 332 genes that were differentially expressed
between the two strains which needed further analysis (Figure 35A). The single base resolution of RNA–Seq experiments also allowed SNP detection to complement the differential expression data. Known SNPs in both Rpe65 (233) and Tyr (234) in A/J and BALB/c but not B6 mice were verified (Figure 36A-P). Moreover, the global scale of expression data from RNA–Seq allowed re-examination of previous QTL studies to provide new insights. For example, in the QTL location on chromosome 10 proposed to account for the difference in cone photoreceptor numbers between A/J and B6 mice (236), a 2–fold differential expression of only one gene, Enpp3, that encodes ectonucleotide pyrophosphate/phosphodiesterase 3 was found. Interestingly Enpp3 contained a SNP in the protein–coding sequence leading to a proposed amino acid residue change from Asn to Ser (Figure 36Q-V). SNP analysis of other differentially expressed genes (Table 6), however, did not reveal any further molecular basis for the age–related phenotype in A/J mice. This prompted the use of pathway analysis software to define perturbations in cellular pathways across the differing mouse genetic backgrounds.

3.3.4 Pathway analysis highlights inflammatory priming coupled with impaired retinal homeostatic cellular pathways in 1–month–old A/J mice before retinal pathology is evident

RNA–Seq of whole eyes from 1–month–old mice with the three genetic backgrounds revealed genes that were exclusively expressed at a higher level by at least 2–fold ($p \leq 0.05$) in each strain with respect to the other two strains as well genes expressed at a higher level in two different strains as compared with the third strain. The analysis identified 133, 164, and 235 genes with higher expression by at least 2–fold
(p<0.05) exclusively in either A/J, BALB/c or B6 mice, respectively. We also found shared increased expression of 52 genes between A/J and BALB/c mice, 26 genes between A/J and B6 mice, and 56 genes between BALB/c and B6 mice (Figure 35B). Ingenuity pathway analysis of these genes revealed that A/J mice exhibited preferentially higher expression of a large cohort of inflammatory gene products, a subset of which was also shared with BALB/c mice. Genes with preferentially decreased expression in A/J compared to BALB/c and B6 mice included those encoding proteins in cellular pathways involved in maintaining normal retinal homoeostasis and protecting against tissue damage. In contrast to A/J mice, BALB/c and B6 mice exhibited overlapping increased expression of a cohort of retinal homeostatic genes as well as exclusive strain-specific increased expression of such genes. Furthermore, there was selective increased expression of key immune regulatory genes in BALB/c mice that could counteract the effects of inflammatory genes found to be elevated in common with A/J mice. Taken together, these network expression patterns present at 1–month of age in A/J mice indicate that the normal appearing retina was primed for inflammation with an absence of key homeostatic and immune protective molecules. This environment could therefore preferentially trigger severe ARD in A/J mice. This phenomenon was investigated with increasing age and stress to the retina.

### 3.3.5 Inflammatory priming in A/J retina is exacerbated with age

Ingenuity pathway analysis revealed an activated inflammatory priming pathway in young A/J and BALB/c mice centered on interferon (IFN) signaling. This was evidenced by increased expression of inflammatory gene products such as interferon regulatory factor 7 (Irf7), the target of interferon–induced signaling, Stat1, and Stat1–inducible markers of
inflammation such as guanylate–binding protein 1 (Gbp1). Exhibiting markedly increased expression in young 1–month–old A/J mice (Figure 37A), expression of this entire network was further exacerbated with age as evidenced by increased expression of Gbp1, Irf7, and Stat1 assessed by RT–PCR in 8–month–old A/J mice. There was minimal age–related elevation of expression detected in B6 mice over the same period (Figure 37B). This inflammatory network was also elevated in BALB/c mice as compared to B6 mice (Figure 37C), albeit at a lower level than in A/J mice. Moreover, BALB/c mice did not exhibit the marked age–related increase in Gbp1, Irf7, and Stat1 expression from 1–to 8–months of age (Figure 37D). This difference indicated that the inflammatory primed network in A/J mice transitions to a chronic state of inflammation with age as a result of other factors, possibly involving RPE cells, key regulators of retinal inflammation.

3.3.6 Marginally–expressed retinal homeostasis proteins exhibit abnormal RPE localization in A/J mice

A large cohort of genes with preferentially decreased expression in A/J compared to BALB/c and/or B6 mouse eyes are involved in homeostatic maintenance of the retinal environment. These genes encode diverse groups of proteins such as those in the glutathione S–transferase (GST), glutathione peroxidase (GPX), heat shock protein (HSP) and metallothionein (MT) families (Figure 38A). Expression of genes potentially involved in phagosomal processing such as Arsi (237), Atp6v0c (238), and Mcoln3 (239), and key genes in immune regulation such as Socs1 (240) were also decreased in A/J mouse eyes (Figure 38A). IHC staining revealed that expression levels in mouse retinas correlated strongly with transcript abundance identified by RNA–Seq in mouse eyes. Examination of the cone marker, phosphodiesterase–6c, PDE6C, illustrated the increased cone numbers in B6 as
compared to A/J mice (Figure 38B). Because many of the highlighted genes play important roles in RPE processing and response to oxidative stress (241), the localization of markers like mucolipin 3, MCOLN3, (figure 38C) and glutathione peroxidase 3, GPX3, (Figure 38D) were examined. Both displayed dim signals in A/J RPE, but much stronger signals in B6 RPE. The MCOLN3 staining distribution also suggested that this protein extends to RPE cell processes that interact with the photoreceptor layer. IHC staining of myosin VIIa (MYO7A), a well known component of the photoreceptor cilium involved in RPE−mediated protein movement and photoreceptor phagocytosis (88, 242), provided only weak signals in the A/J inner segment and RPE cell layer, whereas staining was more pronounced in both these layers of the B6 retina (Figure 38E). Attenuated expression of MYO7A can contribute to mis−localization of key photoreceptor and RPE proteins, such as rhodopsin (RHO) and RPE65. This was evidenced by a RHO signal in the inner segments of A/J retinas, which was absent in B6 retinas (Figure 38F) and the heterogenous RPE65 signal in RPE cells of A/J but not B6 mice at 8-months of age (Figure 30L). Moreover, staining retinas for reactive oxygen species (ROS) with the marker dihydroethidium (DHE), revealed an enhanced signal in 8−month−old A/J retinas before light exposure that became even more pronounced after this procedure. Only a background signal was apparent in BALB/c and B6 retinas (Figure 39). Furthermore, staining for bone morphogenic protein 4 (BMP4), a marker for oxidative stress−induced RPE senescence (243), produced a more enhanced signal in A/J RPE and inner segments of cone photoreceptors than in B6 retina at 1−month of age (Figure 38G).

3.4 Discussion and conclusions
Phenotypic variability based on genetic background differences observed in mice (140, 244) can provide models for complex human conditions and explain the variable susceptibility to certain conditions among different individuals with the same allelic variant of disease. With no reliable markers to date for ARD before it becomes clinically evident, genetic studies have involved only affected individuals, making it difficult to identify factors that trigger this condition. Compared to BALB/c and B6 mice, A/J mice displayed accelerated retinal degeneration stemming from differences in their natural genetic background. Declining visual function with age associated with worsening RPE and photoreceptor cell pathology was documented in these mice. Given that the retina is fully developed after 1 month in mice, a comprehensive set of approaches were used to identify genetic factors in 1–month–old mice that lead to severe ARD at 8-months in A/J but not BALB/c or B6 mice.

Identifying global gene variations associated with complex diseases of multi–genic origin can be challenging. Current studies noted that CSS panels, though capable of revealing some complex disease associations (235, 245), lack the breadth to identify distant interacting genetic factors characteristic of a complex disease like ARD. RNA–Seq allows a more complete documentation of genetic changes relevant to complex disease etiology (246). This is especially true for age–related neurodegenerative conditions, as illustrated by the recent discovery of two novel genetic loci associated with Alzheimer’s disease by high–throughput sequencing methodology (247). The challenge of RNA-Seq technology lies in the large–scale translation of genetic variations into knowledge about the molecular pathogenesis of complex diseases. By studying cellular pathways encoded by these genes as integrated systems one can begin to understand their
derived phenotypes (248) and identify genes that critically contribute to complex disease traits. In this set of experiments, RNA-Seq of young mouse eye tissue across different genetic backgrounds was performed before significant pathology was present. This analysis revealed global gene expression changes that identified factors that apparently predispose A/J mice to a severe age-related retinal degenerative phenotype, as well as factors in BALB/c and B6 mice that prevent this pathology. Complementing morphologic assessment of ARD with deep sequencing technology allowed the identification of early signatures of disease in inbred mice with different genetic backgrounds.

RPE cells are critical for the maintenance of the blood/retina barrier and retinal neurons and must possess mechanisms that protect the retina against oxidative stress generated by exposure to light and high oxygen tension. Compared to BALB/c and B6 mice, A/J mice displayed decreased expression of key genes needed by RPE cells to mitigate such stress. Abundant in the retina, the GPX family encodes a critical group of retinal detoxification enzymes, and Gpx3 expression was found to be markedly decreased in A/J mice with GPX3 largely localizing to RPE cells. Inadequate activity of antioxidant enzymes like GPX in other organ systems has been implicated in the progression to chronic inflammatory pathology (249) and polymorphisms of Gpx3 have been implicated in human phenotypes of ARD (250, 251). This finding extends to other protein families such as the HSPs, of which expression of both Hspa8 and Hspb1 was decreased in A/J mice. In addition to chaperone mediated activity (252), HSPs combat oxidative stress by increasing levels of glutathione and modulating the redox status of cells (253). Decreasing levels of glutathione and associated proteins with age (254, 255) coupled with constitutively decreased expression of these important homeostatic gene families in
young A/J compared to BALB/c and B6 mice offer an explanation for the accelerated age–related degenerative phenotype evident in A/J mice. Pathologic changes seen in A/J RPE, characterized by cell swelling and build–up of undigested disc elements, have also been reported in albino and pigmented rats deprived of selenium (256, 257), a critical co–factor for activation of antioxidant proteins such as GPX3. Inadequate protection against oxidative stress was also accompanied by accelerated loss of photoreceptors, especially in the central retina of these rats, suggesting that oxidative damage to RPE cells may initiate the detrimental process and that photoreceptor loss is a secondary effect. An inadequate oxidative stress response thus could produce wide–ranging adverse effects in the retina. One detrimental age–related effect, namely cellular senescence, was evidenced by increased BMP4 expression in A/J mice as young as 1–month of age, and this could lead to secretion of various factors that further fuel chronic inflammation (258).

The RPE is a central site of immune regulation in the retina (259). Moreover, constant stress and cellular senescence can change homoeostatic set points and exacerbate immune responses leading to chronic inflammation and disease progression (260). Though A/J mice have natural deficiencies of complement (C5a) and NOD (Naip5) genes involved in susceptibility to bacterial/fungal infection, recombinant congenic strains (261, 262) have shown that these genes are controlled by additional factors and have little impact on the inflammatory priming pathways highlighted. In young A/J and to a lesser degree BALB/c but not B6 mice, RNA–Seq revealed an inflammatory-primed network characterized by increased interferon gene products such as Stat1 and its downstream effectors (240). The inflammatory-primed state identified in
A/J mice is not limited to the retina, as it also likely contributes to the enhanced A/J inflammatory response in experimental allergic asthma (263). Moreover the graded retinal inflammatory changes evident from A/J to BALB/c to B6 mice are consistent with studies of allergic airway inflammation in the lungs of these strains (264). These studies indicate that A/J mice are most susceptible to inflammation in tissues of high oxygen tension (retina and lungs), further implicating an inadequate oxidative stress response as a driving force of late stages of inflammatory disease progression. The inflammatory–primed state of the retina in A/J mice was compounded by markedly lower levels of immune regulatory enzymes. Compared to BALB/c mice, A/J mice exhibited decreased expression of genes such as Tyro3 and its downstream effector Socs1 that serve to control this inflammatory response (265). Moreover, inadequate function of enzymes like GPX3 would lead to increased oxidative stress and hydrogen peroxide production, which reduce the expression and function of complement factor H (266), a key complement regulatory enzyme implicated in disease pathogenesis (267). Thus, the inflammatory–primed state of the retina in young A/J mice reflects a para–inflammatory state (268) that, accentuated by increased oxidative stress, transitions to chronic inflammation and cellular dysfunction with increasing age (Figure 40), just as observed in diseased patients (269). Priming of RPE cells can translate to secondary effects on critical cellular processes such as phagocytosis and digestion of POS, which are increasingly affected in A/J mice with age. Another secondary effect is the production of anti-retinal antibodies to α-crystallin, GFAP, and α-enolase found in sera of ARD patients (270). Preferential activation of Stat1 has been detected in several autoimmune/inflammatory conditions (240), and this increased significantly and
exclusively in A/J mice with age. We also noted increased GFAP expression with age and α-enolase was elevated at the transcript level in A/J mice as early as 1-month of age. The RPE/retina barrier function is most detrimentally affected when both oxidative stress and immune activation are induced (271), suggesting that these additive effects of aging in A/J mice could potentially lead to auto-immune responses. Ocular immune privilege is normally responsible for the impaired tolerance to retinal antigens (272), but exposure to such antigens upon age-related barrier compromise could further contribute to disease progression.

Preliminary RNA-Seq analysis of whole eye tissues from both nocturnal (Long-Evans rat) and diurnal (Nile rat) rodent species at a young age revealed gene signatures most closely related to B6 mice and clearly distinct from A/J mice (Table 7). This observation emphasizes that these new findings extend beyond model mouse species and can provide an additional frameworks for understanding etiologic factors contributing to complex age-associated diseases in higher organisms. This is especially relevant to human blinding conditions associated with aging such as age-related macular degeneration (AMD), the leading cause of blindness in the industrial world and now designated as one of the major blinding diseases world-wide (273). The complex etiology of AMD is reflected by relative paucity of effective compounds for its early prevention and treatment with the main risk factor being increasing age. Complex trait analysis of genome wide association studies having identified the vast majority of variations in non-coding regions (274) and the recent identification of long intervening non-coding RNAs implicated in controlling a diverse set of biological processes (275, 276) that include differentiation of the murine retina (277) together with acceleration of
their discovery by application of next generation sequencing technology (278), could serve as the next frontier to facilitate understanding of complex disease phenotypes.

In conclusion, RNA-Seq of 3 inbred background strains of mice with differing susceptibilities to ARD elucidated genes encoding components of cellular pathways that contribute to this blinding condition at a young age before phenotypic changes are apparent. Relatively high expression of pro-inflammatory factors coupled with low expression of homeostatic protective factors such as those involved in oxidative stress response were identified and then found to localize to the RPE layer. This combination makes A/J mice especially vulnerable to rapidly progressive ARD relative to either BALB/c or B6 mice. A/J mice reared in the dark still developed ARD, illustrating that the pathogenic mechanisms described here differ from those involving excessive accumulation of the all-\textit{trans}-retinal chromophore (279). These results, in conjunction with imaging techniques that demonstrated elevated level of ROS and changes in phagocytotic processing in the RPE, allow a more comprehensive understanding of a complex neuronal degeneration. With advances in phenotypic and genotypic characterization technology, methodologies outlined in this set of experiments represent a powerful paradigm to unveil cellular changes that trigger and drive progression of complex neurodegenerative diseases extending beyond the eye.
**Table 5.** GoTerm breakdown of transcript reads across different RNA–Seq experiments with from 1-month-old A/J, BALB/c and B6 mice whole eye tissue.

<table>
<thead>
<tr>
<th>Go Term</th>
<th>A/J Eye</th>
<th>BALB/c Eye</th>
<th>B6 Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autophagy</td>
<td>28 (0.22)</td>
<td>27 (0.21)</td>
<td>28 (0.22)</td>
</tr>
<tr>
<td>Binding activity</td>
<td>1,077 (8.54)</td>
<td>1,055 (8.38)</td>
<td>1,083 (8.44)</td>
</tr>
<tr>
<td>Biogenesis</td>
<td>94 (0.75)</td>
<td>95 (0.75)</td>
<td>96 (0.75)</td>
</tr>
<tr>
<td>Catalytic activity</td>
<td>178 (1.41)</td>
<td>181 (1.44)</td>
<td>182 (1.42)</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>306 (2.43)</td>
<td>307 (2.44)</td>
<td>327 (2.55)</td>
</tr>
<tr>
<td>Cell cycle</td>
<td>337 (2.67)</td>
<td>321 (2.55)</td>
<td>326 (2.54)</td>
</tr>
<tr>
<td>Cell death</td>
<td>185 (1.47)</td>
<td>185 (1.47)</td>
<td>189 (1.47)</td>
</tr>
<tr>
<td>Cytoskeleton organization</td>
<td>172 (1.36)</td>
<td>170 (1.35)</td>
<td>173 (1.35)</td>
</tr>
<tr>
<td>Developmental process</td>
<td>185 (1.47)</td>
<td>192 (1.53)</td>
<td>196 (1.53)</td>
</tr>
<tr>
<td>DNA repair</td>
<td>144 (1.14)</td>
<td>143 (1.14)</td>
<td>144 (1.12)</td>
</tr>
<tr>
<td>Homeostatic process</td>
<td>113 (0.90)</td>
<td>114 (0.91)</td>
<td>116 (0.90)</td>
</tr>
<tr>
<td>Metabolic process</td>
<td>1,886 (14.96)</td>
<td>1,862 (14.79)</td>
<td>1,897 (14.78)</td>
</tr>
<tr>
<td>No term</td>
<td>2,456 (19.48)</td>
<td>2,483 (19.72)</td>
<td>2,510 (19.58)</td>
</tr>
<tr>
<td>Protein folding</td>
<td>97 (0.77)</td>
<td>98 (0.78)</td>
<td>99 (0.77)</td>
</tr>
<tr>
<td>Protein modification</td>
<td>456 (3.62)</td>
<td>456 (3.62)</td>
<td>464 (3.62)</td>
</tr>
<tr>
<td>Regulatory process</td>
<td>263 (2.09)</td>
<td>261 (2.07)</td>
<td>263 (2.05)</td>
</tr>
<tr>
<td>RNA processing</td>
<td>362 (2.87)</td>
<td>363 (2.88)</td>
<td>364 (2.84)</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>1,254 (9.95)</td>
<td>1,256 (9.98)</td>
<td>1,297 (10.11)</td>
</tr>
<tr>
<td>Structural molecule activity</td>
<td>103 (0.82)</td>
<td>103 (0.82)</td>
<td>106 (0.83)</td>
</tr>
<tr>
<td>System process</td>
<td>89 (0.71)</td>
<td>85 (0.68)</td>
<td>82 (0.64)</td>
</tr>
<tr>
<td>Transcription</td>
<td>1,213 (9.62)</td>
<td>1,215 (9.65)</td>
<td>1,230 (9.59)</td>
</tr>
<tr>
<td>Translation</td>
<td>268 (2.13)</td>
<td>281 (2.23)</td>
<td>283 (2.21)</td>
</tr>
<tr>
<td>Transport</td>
<td>1,340 (10.63)</td>
<td>1,336 (10.61)</td>
<td>1,374 (10.71)</td>
</tr>
<tr>
<td>Total transcripts (&gt; 1 FPKM)</td>
<td>12,606</td>
<td>12,589</td>
<td>12,832</td>
</tr>
</tbody>
</table>

All transcripts detected at a level of 1 FPKM (average of three biological replicate runs) in A/J, BALB/c, and B6 whole eye samples were subjected to GoTerm categorization with Amigo v1.8. Numbers in parenthesis indicate the percentage of transcripts that fell into the listed category.
Table 6. Differential expression profile of genes in the A/J and B6 mouse eye and SNP analysis of differentially expressed genes. A total of 332 genes were differentially expressed by at least 2−fold (p<0.05) in A/J and B6 mouse eyes. The breakdown of fold differences is listed. SNPs in these sets of genes are also listed with coding region SNPs classified as synonymous, non−synonymous or untranslated region (UTR). UTR SNPs are further identified as either 3’ or 5’ SNPs. Of the 393 coding SNPs identified, 75 were not found in the dbSNP database encompassing 37 different genes.

<table>
<thead>
<tr>
<th></th>
<th>A/J Eye</th>
<th>B6 Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher in expression by &gt;2 fold</td>
<td>117</td>
<td>215</td>
</tr>
<tr>
<td>&gt;5−fold</td>
<td>27</td>
<td>33</td>
</tr>
<tr>
<td>3−5−fold</td>
<td>25</td>
<td>60</td>
</tr>
<tr>
<td>2−3−fold</td>
<td>65</td>
<td>132</td>
</tr>
<tr>
<td>Genes with SNPs</td>
<td>47</td>
<td>58</td>
</tr>
<tr>
<td>Total SNPs</td>
<td>232</td>
<td>229</td>
</tr>
<tr>
<td>Coding Region SNPs</td>
<td>182</td>
<td>211</td>
</tr>
<tr>
<td>Synonymous SNPs</td>
<td>66</td>
<td>62</td>
</tr>
<tr>
<td>Non−Synonymous SNPs</td>
<td>40</td>
<td>23</td>
</tr>
<tr>
<td>UTR SNPs</td>
<td>76</td>
<td>126*</td>
</tr>
<tr>
<td>3’ UTR SNPs</td>
<td>71</td>
<td>122</td>
</tr>
<tr>
<td>5’ UTR SNPs</td>
<td>5</td>
<td>3</td>
</tr>
</tbody>
</table>

*One of these UTR SNPs lies at a predicted splice site.

SNP detection and analysis was done with the GenomeStudio RNA Sequencing Module (Illumina). Allelic base calls for A/J and BALB/c mice were analyzed for those that differed (Call type: Diff) from the reference base (C57BL/6). Only different base calls that were common to all three different biological replicate sample lanes were considered as SNPs for further analysis. SNPs were analyzed to see if they fell within exon boundaries of genes of interest. Coding region SNPs were further characterized as either synonymous, non−synonymous, or UTR based on SNPs noted in NCBI dbSNP Build
128 for mus musculus. If SNPs were not found in the database, they were manually examined with the University of California Santa Cruz mouse genome assembly (mm9).
Table 7. Transcript reads (FPKM) of selected genes from A/J, BALB/c, and B6 mice as well as from Long-Evans rat and Nile rat eyes. The three inbred mouse models were compared to two different rodent species with varying photoreceptor populations: the nocturnal Long-Evans rat, which possesses a large rod population and the diurnal Nile rat, which has a large percentage of cone photoreceptors. Selected examination of photoreceptor genes revealed the relative rod dominance in the Long-Evans rat (increased Rho expression) and the cone dominance in the Nile rat (increased Opn1mw expression). Examination of selected retinal homeostasis and inflammatory genes revealed that Long-Evans and Nile rats possess retinal environments distinct from A/J mice that are most similar to B6 mice.

### Rod and Cone Photoreceptor Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>A/J</th>
<th>BALB/c</th>
<th>B6</th>
<th>Long–Evans rat</th>
<th>Nile rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opn1mw</td>
<td>54.68</td>
<td>44.36</td>
<td>62.97</td>
<td>36.38</td>
<td>162.00</td>
</tr>
<tr>
<td>Rho</td>
<td>4365.69</td>
<td>5389.12</td>
<td>61.62</td>
<td>9831.11</td>
<td>1918.14</td>
</tr>
</tbody>
</table>

### Retinal Homeostasis Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>A/J</th>
<th>BALB/c</th>
<th>B6</th>
<th>Long–Evans rat</th>
<th>Nile rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmp4</td>
<td>17.57</td>
<td>15.06</td>
<td>10.62</td>
<td>11.36</td>
<td>8.20</td>
</tr>
<tr>
<td>Gpx3</td>
<td>29.6</td>
<td>82.90</td>
<td>205.55</td>
<td>77.18</td>
<td>7.00</td>
</tr>
<tr>
<td>Hspb1</td>
<td>62.85</td>
<td>139.35</td>
<td>116.38</td>
<td>376.04</td>
<td>288.30</td>
</tr>
<tr>
<td>Myo7a</td>
<td>1.83</td>
<td>2.71</td>
<td>5.48</td>
<td>1.35</td>
<td>3.02</td>
</tr>
</tbody>
</table>

### Inflammatory Genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>A/J</th>
<th>BALB/c</th>
<th>B6</th>
<th>Long–Evans rat</th>
<th>Nile rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ifi44</td>
<td>12.90</td>
<td>5.50</td>
<td>0.60</td>
<td>3.77</td>
<td>2.82</td>
</tr>
<tr>
<td>Irf7</td>
<td>28.95</td>
<td>27.81</td>
<td>16.16</td>
<td>28.82</td>
<td>5.40</td>
</tr>
<tr>
<td>Stat1</td>
<td>14.06</td>
<td>10.56</td>
<td>10.35</td>
<td>19.27</td>
<td>11.22</td>
</tr>
<tr>
<td>Socs1</td>
<td>0.97</td>
<td>2.13</td>
<td>1.76</td>
<td>2.78</td>
<td>1.82</td>
</tr>
</tbody>
</table>

Long–Evans rat and Nile rat libraries were sequenced using paired-end sequencing using the Illumina GA IIx. Long–Evans rat data were aligned with University of California
Santa Cruz rat genome assembly (rn4). Preliminary Nile rat data were assembled de novo by using Velvet and Oases software.
**Figure 25.** A/J mice display a pronounced age–dependent decline in vision. (A) Light microscopy of A/J mouse retinas reveals a marked decrease in outer nuclear layer (ONL) and inner nuclear layer (INL) thickness in 8–month–old as compared to 1–month–old animals as well as pathological changes in the RPE layer at 8-months of age, with the boxed area shown at higher magnification (pyknotic cell indicated by black arrow). These changes were minimal in B6 mice. (B) ONL thickness plotted as a function of distance from the optic nerve head (ONH; in millimeters) shows that most pronounced decline occurs between 3– and 8–months of age, a finding absent in B6 mice. (C) Cone cell sheaths were imaged by PNA staining (left panels) at 1–, 3–, and 8–months of age. Average numbers of cone cells in both the superior and inferior retina in a 100 µm range located 500 µm away from the ONH were plotted (right panel, A/J in red, B6 in black) to show a marked decline between 3– and 8–months of age in A/J mice. (D) Representative ERG responses at 1.6 log cd·s·m⁻² are shown in the left panel and functional a–wave and b–wave amplitudes obtained from A/J and B6 mice at 1–, 3–, and 8–months of age are plotted in the right panel (A/J in red, B6 in black). ERG responses were more attenuated with age in A/J as compared to B6 mice under both scotopic and photopic conditions (p<0.001). Scale bars in panels A and C are 20 µm.
Figure 26. A/J and B6 mouse eyes exhibit different retinoid content profiles. (A) Normal–phase HPLC separation of nonpolar retinoids from retinal homogenates of 1−, 3−, and 8−month−old A/J and B6 mouse eyes showed decreased levels of both 11− cis−retinal (top) and all−trans−retinyl esters (bottom) in A/J (red symbols) relative to B6 (black symbols) mice. These differences were most pronounced at 8−months of age (n>8). (B) Fundus autofluorescence (AF) was measured by SLO (485 nm excitation; emission filter, 500−700 nm) at 1−and 8−months of age. The intensities of AF plotted as mean gray values revealed a 4−fold increase in A/J mice at 8−months of age as compared to 1−month of age whereas only a mild age−related increase of AF intensity was noted in B6 mice. A2E, one of the main fluorophores in the retina, was extracted from 1−and 8−month−old mouse eyes and quantified by normal phase HPLC. Amounts of A2E increased with age in both A/J and B6 mice but did not correlate with the degree of fundus AF. (C) In both A/J (left panel) and B6 (right panel) mice, the retinoid cycle was functional at 1.5−months of age and the 11− cis−retinal regeneration ratio in A/J mice after a light stimulus (10000 cd·m−2, less than 2 ms) was 2−fold higher than in B6 mice. This is consistent with the increased expression of RPE65 in A/J mice. B, C: n>3 at each time point. Bars indicate standard deviation (SD) in all panels.
Figure 27. Age–related degenerative changes in eyes of A/J mice are independent of lighting conditions. (A) Thicknesses of the ONL plotted as a function of distance from the optic nerve head (ONH; in mm) in retinas of 8-month-old A/J (red circles) and B6 (black circles) mice maintained in the dark illustrate that age–related changes in A/J mouse retina does not depend on exposure to light (n>4). (B) Plots of functional a–wave amplitudes under scotopic condition and b–wave amplitudes under photopic conditions obtained from A/J and B6 mice at 8–months of age are shown. ERG responses were attenuated in A/J relative to B6 mice under both scotopic and photopic conditions (n>4, P<0.001). Bars indicate SDs.
**Figure 28.** Mild progression of age–dependent retinal dysfunction in BALB/c mice. (A) In vivo SD–OCT imaging of retinal cross–sections from BALB/c and B6 mice reveals a mild decrease in outer nuclear layer (ONL) thickness in BALB/c as compared to B6 mice at 8–months of age. (B) Cone cell sheaths in retinal cross sections were imaged by PNA staining (left panels) and numbers of cone cells were counted in both the superior and inferior retina over a 100 µm range located 500 µm away from ONH and the average number was plotted (right panel). Numbers of cone cells were comparable in BALB/c mice and B6 mice at 8–months of age. (C) Representative ERG responses at 1.6 log cd·s·m$^{-2}$ are shown in the left panels and functional a–wave amplitudes under scotopic conditions and b–wave amplitudes under photopic conditions obtained from BALB/c and B6 mice at 8–months of age were plotted (right panel). ERG responses were attenuated in BALB/c relative to B6 mice under scotopic conditions (P<0.001) whereas amplitudes were comparable between the two strains under photopic conditions, consistent with the results illustrated in panels A and B. (D) Amounts of the visual chromophore, 11–cis–retinal, in the eye, which are comparable to those of visual pigments, were quantified by HPLC at 8–months of age. Amounts of 11–cis–retinal in the BALB/c mouse eye were 80% of those found in the B6 mouse eye. Three to four animals were evaluated for each experimental condition. ONL, outer nuclear layer. Scale bars are 40 µm in panel A and 20 µm in panel B. Bars indicate SDs in all panels. Asterisks indicate statistically significant differences.
Figure 29. Increased retinal autofluorescence in A/J mice with age relates to inflammatory changes and immune cell infiltration. (A) Representative SLO autofluorescent images of the outer retina (480 nm excitation, emission filter, 500–700 nm) of 1− and 8−month−old A/J and B6 mice are shown. A/J mice exhibited increased autofluorescent spots with age, which were negligible in B6 mice (n>3). (B) SD−OCT imaging of A/J and B6 mice at 1− and 8−months of age revealed infiltrating cells only in the subretinal space of 8−month−old A/J mice (red arrows in left panel) that were also observed in plastic block sections (black arrows in right panel). Thickness of ONL in OCT images is indicated by arrows to highlight the decline in A/J mice at 8-months of age compared to B6 mice. (C) The cellular infiltration seen by OCT and in plastic sections was of inflammatory origin as evidenced by increased Iba−1 staining (activated microglia cells) and increased GFAP staining (activated Müller glial cells and astrocytes) in 8−month−old compared to 1−month−old A/J retinas. These age−related changes were absent from 8−month−old B6 mouse retina. Labeled retinal layers are as follows: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer; PR, photoreceptor layer. Scale bars in all panels are 50 μm.
**Figure 30.** Significant pathological changes are apparent in RPE cells of A/J mice before measurable visual decline. (A) TEM imaging of 3-month-old A/J retinas reveals RPE cells with an average width of 31.0 µm with abnormal disc membrane accumulations (black arrows). (B) Ingested phagosomes in A/J mice (C) show undigested accumulations. (D) Phagosomes are also disrupted with (E) phagocytotic material exposed to the cytoplasm. (F) TEM imaging of B6 RPE cells reveals an average width of 23.0 µm with (G) uptake of discs that (H) are normally processed. (I) Ingested discs traffic normally with (J) membrane enclosed disc elements clearly visible. These findings are supported by (K) ex vivo TPM imaging of A/J and B6 RPE cells at 1−, 3−, and 8−months of age. Dysmorphic features in 3−month-old A/J mice were exacerbated in 8-month-old A/J mice, but were absent in B6 mice. (L) RPE65 provided a localized uniform signal in 1−month−old A/J mouse retina but a decreased and heterogeneously localized signal in 8−month−old A/J mouse retina (yellow arrows). No apparent age−related changes were noted in retinas of B6 mice. (M) Regeneration of 11−cis−retinal decreased significantly (75%) in A/J retinas by 8−months of age whereas no such change was noted in B6 mice. Labeled layers of the retina are as in Figure 29. Scale bars are 5 µm in panels A and F; 1 µm in panels B, D, G, and I; 0.5 µm in panels C, E and H, J; and 20 µm in panels K and L.
Figure 31. Increased autofluorescence in BALB/c mice with age occurs without obvious pathological changes in the RPE. (A) Representative SLO autofluorescent images are shown of BALB/c and B6 mouse outer retina (485 nm excitation; emission filter, 500–700 nm). BALB/c mice exhibited age-related (1– to 8–months) increased autofluorescence and punctate white dots not seen in B6 mice (n>3). (B) Compared to B6 mouse retina, 8–month–old BALB/c mouse retina featured mild cellular infiltration resulting from inflammatory and immune cell activation evidenced by Iba−1 staining (activated microglia cells) and GFAP staining (activated Müller glial cells and astrocytes). Slight staining of Iba−1, indicated by arrows, was not recognized by the GFAP stain. (C) The size and morphology of RPE cells from 8–month–old BALB/c and B6 mice were imaged by TPM. No obvious pathological changes were detected in the RPE of BALB/c mice and RPE cell sizes were comparable to those of B6 mice (n>50). Labeled layers of the retina are: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IS, inner segment; OS, outer segment; RPE, retinal pigmented epithelium. Scale bars are 40 µm in panel A, 50 µm in panel B, and 20 µm in panel C. Bars indicate SDs in panel C.
**Figure 32.** Genetic panel study reveals no significant phenotypic changes in B6 mice with single A/J chromosome substitutions. A chromosome substitution strain (CSS) panel was used in which single chromosomes (labeled 1–19, X, Y and Mito (mitochondrial DNA)) from A/J mice were substituted into the B6 background. (A) An example of the genetic makeup of chromosome 8 substituted mice (Chr 8), which only possess chromosome 8 from A/J whereas other chromosomes are from B6 mice. No significant functional or structural changes were observed in 8–month–old mice when either (B) 11–cis–retinal levels or (C) nuclei numbers in the outer nuclear layer were compared in all CSS strains relative to B6 mice. Representative (D) HPLC chromatograms and (E) plastic block sections of A/J, B6 and Chr 8 mice are shown. These data indicate a multi–genic contribution to the age–related abnormal retinal phenotype observed in A/J mice. ONL, outer nuclear layer. Scale bars in panel E are 40 μm.
Figure 33. RNA-Seq of biological replicates of 1-month old eye tissue from A/J, BALB/c and B6 mice reveals high reproducibility of individual background runs. For mice of each genetic background (A/J, BALB/c, and B6), three biological replicate eye samples were run on individual lanes of an Illumina Genome Analyzer IIx. (A) A plot of the three biological replicate runs for A/J mouse eyes showed little variability. (B) Most reads from biological replicate runs fell along the line representing equal expression, with a high correlation coefficient of 0.98. Similarly, a plot of the three biological replicate runs for BALB/c mouse eyes, (C) and (D), and B6 mouse eyes, (E) and (F), also displayed the same equal expression with correlation coefficients of 0.98 and 0.96, respectively. The overall strong correlation of biological replicate runs indicates that any differential expression between genetic backgrounds did not result from sequencing errors. Moreover, use of three biological replicates for each genetic background allowed statistical calculations to remove outlier differences.
**Figure 34.** RNA–Seq data are verified by RT–PCR and immunoblotting. (A) RT–PCR validates the differential expression profile detected by RNA–Seq with different probes for genes exhibiting either high or low differential expression compared to those with virtually equal expression in eyes of A/J and B6 mice. Dark gray bars represent the RNA–Seq values and light gray bars, the RT–PCR values. (B) The correlation between the RT–PCR and RNA–Seq findings was extremely high ($R^2=0.99$). (C) RNA–Seq changes also predicted selected protein level changes assessed by immunoblots of the common visual cycle proteins GNAT1, LRAT, RHO and RPE65. Protein separation was performed on 12% SDS–PAGE gels. Immunoblotting (Immobilon–P polyvinylidenedifluoride; Millipore) was carried out according to standard protocols. Alkaline phosphatase–conjugated goat anti–mouse IgG or goat anti–rabbit IgG (Promega) were used as secondary antibodies. Protein bands were visualized with 5–bromo–4–chlooro–3–indolyl phosphate/nitro blue tetrazolium color development substrate (Promega). Dark gray bars represent the immunoblot intensities and light gray bars the RNA–Seq transcript level changes.
**Figure 35.** RNA–Seq of three individual biological replicates of 1-month-old A/J, BALB/c and B6 mouse eyes reveals differential transcriptome profiles. (A) A plot of log fragment per kilobase per million reads (FPKM) from A/J and B6 runs (left) illustrates that the most highly expressed transcripts common to both A/J and B6 eyes are the lens crystallin genes (outlined) and rod photoreceptor genes such as *Gnat1*, *Rho*, and *Sag*. Most genes highly differentially expressed in the A/J eye relate to inflammation (indicated in red), whereas genes with the lowest differential expression in the A/J eye encode homeostatic proteins (indicated in black). The Venn diagram (right) indicates that 12,672 genes have similar expression whereas 332 are differentially expressed by at least 2–fold (p≤0.05) between A/J and B6 eyes. (B) Examination of all three mouse eye transcriptomes reveals those genes that are exclusively more highly expressed in a single mouse strain as compared to the other two mouse strains and those that share increased expression with respect to a third mouse strain. For example, 235 genes are exclusively more highly expressed in the B6 eye while it also shares 26 genes also highly expressed in the A/J eye and 56 also higher in the BALB/c eye. Importantly, a large cohort of inflammatory genes is exhibits increased expression in A/J mice. Interestingly, though both A/J and BALB/c eyes share increased expression of inflammatory genes, only BALB/c eye exhibits a counteracting increased expression of retinal homeostatic and immune regulatory genes, either exclusively or shared with B6 eyes.
A

A/J Eye (log FPKM)

B

Retinal Homeostasis: 
Arsi, Gsto1, Mcoln3, Myo7a, Tyro3

Inflammation: 
Ili204, Rab37

Retinal Homeostasis: 
Atp6v0c, Fgl2, Gpx3, Hspa8, Muc1, Vgf

Inflammation: 
Ddx60, Dhx58, Elf2, Gbp3, Gbp4, Ili1, Oasl2, Mx2

Retinal Homeostasis: 
Cntf, Gsta1, Hspb1, Mt1, Mt2

Inflammation: 
Gbp1, Ili27, Ili44, Isg15, Oasl1a, Oasl2, Oasl3, Slfn4

Immune Regulation: 
Mif, Socs1

B6

215

12,672

117

A/J

BALB/c

235

56

164

52

133

26

56

126
Figure 36. Key SNPs are identified by RNA-Seq. In albino A/J and BALB/c mice there is a missense mutation in RPE65 at amino acid 450 that contributes differentially to light−induced retinal damage. We noted increased amounts in (A) A/J and (B) BALB/c mice as compared to (C) B6 mice of (D) the Rpe65 message and also found that the nucleotide change in (E) A/J and (F) BALB/c mice was not evident in (G) B6 mice or the (H) reference assembly that should give rise to this altered protein product. The Tyr transcript is known to be transcribed at similar levels in A/J, BALB/c, and B6 mice, but a single mutation at nucleotide position 308 that causes a missense mutation at amino acid 103 (cysteine to serine) abolishes pigment production in albino mice. We found similar levels in (I) A/J, (J) BALB/c, and (K) B6 mice of (L) this Tyr message. We also observed a mutation at nucleotide position 308 in (M) A/J and (N) BALB/c mice that was not evident in (O) B6 mice or (P) the reference assembly that would give rise to the missense mutation noted. Our expanded analysis to a QTL region in chromosome 10 revealed differential expression of Enpp3 and a SNP corresponding to a missense mutation. Compared to (Q) A/J mice, there was greater expression in (R) B6 mice of (S) Enpp3. Examination of this gene revealed a SNP at position 24,513,440 in (T) A/J mice that gives rise to a missense mutation that was not evident in (U) B6 mice or (V) the reference assembly.
Figure 37. Pathway analysis of RNA–Seq differential expression profiles reveals age–related inflammatory priming in eyes of A/J mice. (A) Pathway analysis with Ingenuity software unveiled an aberrant inflammatory network in A/J mice characterized by priming of interferon (IFN) at 1–month of age evidenced by: increased activation of Irf7 and Stat1; a coordinated increased expression of Stat1–induced secondary response genes, induction of positive regulatory loop genes in the inflammatory process, and expression of genes involved in immune cell activation. Numbers next to each gene represent the RNA–Seq FPKM values from A/J eye (top in red) and B6 eye (bottom in black) with fold differences indicated next to the vertical arrows. RT–PCR of Gbp1, Irf7, and Stat1 (highlighted in red) showed that (B) this inflammatory priming network is preferentially exacerbated in older mice with pronounced increased expression of gene products from 1–month to 8–months of age in A/J relative to B6 mice. (C) BALB/c mice also exhibited features of inflammatory priming at 1–month of age when the same pathways were examined, but these changes were less pronounced than those in A/J mice. Numbers next to each gene represent the RNA–Seq FPKM values from BALB/c eye (top in blue) and B6 eye (bottom in black) with fold differences indicated next to vertical arrows. RT–PCR of Gbp1, Irf7, and Stat1 (highlighted in blue) showed that (D) in BALB/c mice, inflammatory priming was not exacerbated from 1 to 8–months of age, similar to findings in B6 mice.
Figure 38. Homeostatic processing genes with decreased expression in A/J mice display protein expression in RPE and photoreceptor compartments of the retina. (A) Expression of genes of the glutathione S–transferase (GST), glutathione peroxidase (GPX), heat shock protein (HSP), and metallothionein (MT) families, as well as genes involved in phagosomal processing and in immune regulation exhibited markedly decreased expression in A/J as compared to both BALB/c and B6 mice. Numbers next to each gene represent the RNA–Seq FPKM values from A/J (red), BALB/c (blue) and B6 eyes (black) with the fold difference in A/J compared to BALB/c in blue and that compared to B6 in black indicated next to each vertical arrow. Genes with greater expression in BALB/c are in blue rectangles whereas genes with greater expression in B6 are in black rectangles. IHC of 1-month-old A/J and B6 retinas was done with (B) PDE6C, cone cell marker; (C) MCOLN3, involved in lysosomal degradation; (D) GPX3, involved in ROS detoxification; (E) MYO7A, involved in protein trafficking; (F) RHO, visual pigment in rod photoreceptors; and (G) BMP4, marker of RPE cell senescence. MCOLN3, GPX3 and MYO7A staining was much more pronounced in B6 RPE (white arrows). The aberrant nature of the A/J RPE cell was evidenced by mis-localization of RHO as evidenced by signals in both the OS and IS (white arrows) and increased expression of the senescence marker BMP4 in IS and RPE (white arrows) in A/J mice. Layers of the retina are labeled as in Figure 29. Scale bars are 20 µm.
Figure 39. In vivo ROS generation is detected in the RPE of aged A/J mice. Eight–month–old A/J, BALB/c and B6 mice were dark–adapted overnight and treated with the ROS probe, DHE, by intraperitoneal injection 1 h prior to 5,000 lux light exposure for 30 min. DMSO was used as the vehicle control (Vehicle). Dark–adapted mice unexposed to experimental light were included for the DHE probe treatment as well (No Light). Retinas were harvested 3 h after illumination. ROS signals were captured with a fluorescence microscope under identical exposure conditions. DAPI staining revealed a representative orientation of nuclear layers in retinal cross sections. ROS signals were observed in the RPE of A/J mouse retina irrespective of light exposure (red arrows) and from infiltrated inflammatory cells (yellow arrows) located above the RPE cell layer (n>5). ROS signals from inflammatory cells also were noted in light–exposed BALB/c mice whereas non–specific background signals were seen in both BALB/c and B6 mouse retina (n>5). Labeled layers of the retina are as follows: GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigmented epithelium. Scale bars are 50 µm.
Figure 40. Inadequate protection by the RPE from stress drives the retina from an inflammatory–primed state to a chronic disease state. In a normal homeostatic state there is a delicate balance between stress and the resulting tissue response. Stress caused by photoreceptor outer segment accumulation in the RPE from daily ingestion of oxidized photoreceptor discs could result in oxidative damage and inflammation unless modulated by a network of enzymes such as GPX (left panel). Also, activation of inflammatory factors like STAT1 in response to such RPE cell stress is controlled by regulatory factors such as SOCS1 and CFH. In contrast, low or declining levels of protein expression from these complex sets of interconnected gene networks result in inadequate stress protection and inflammatory changes that cause chronic retinal degeneration (right panel). Thus, decreased expression of homeostatic genes like Gpx foster increased oxidative stress and can reduce other protective factors like Cfh. This is compounded by decreased expression of immune regulatory factors that can exacerbate inflammation and drive disease progression. Subtle genetic differences therefore can have profound effects on the predisposition to and pathogenesis of ARD.
CHAPTER 4: THE ROLE OF NON-CODING RNAs IN VISUAL FUNCTION

Portions of this chapter were previously published in:

4.1 Delineating roles of long intergenic non-coding RNAs in the adult retina

4.1.1 Long intergenic non-coding RNAs and their possible physiological roles

Genetic complexity in the ocular transcriptome of the adult retina (280) allows the encoding of a diverse set of protein-coding genes that support vision (281). However, work over the last few decades has revealed that non–coding RNAs can have a profound effect on the cellular transcriptional and translational landscape. Advances in next generation sequencing along with closer examination of the human (282, 283) and mouse (284) genomes have revealed that the mammalian genome encodes thousands of long non–coding RNAs (135) including over 8,000 long intergenic non–coding RNAs (lincRNAs) in the human genome (285). These transcripts are transcribed from genomic loci flanked by two protein coding genes, are over 200 nucleotides long, undergo typical mammalian RNA processing involving 5’ capping, poly–adenylation, and splicing, but have no protein–coding capability (275, 276, 286). LincRNAs reportedly regulate transcription of protein–coding genes by guiding and tethering chromatin modifying complexes to specific genomic loci in a trans–regulatory manner (275, 287, 288), but the precise mechanisms are still largely unknown as lincRNAs have also been shown to work in a cis–regulatory manner (289). Moreover, lincRNAs show a broad sub–cellular compartmentalization (290, 291) implying that their functional contribution may occur at both the transcriptional and post–transcriptional levels. With increasing association of long non–coding RNAs with human disease (292-294) and recent evidence of lincRNAs related to Mendelian disorders and neurodevelopmental disabilities (295), there is a pressing need to understand the cellular roles of these molecules.
An importance of lincRNAs is supported by their highly tissue-specific expression (286) and location close to protein-coding genes associated with development and transcriptional regulation (278, 289). Model organisms in which to study lincRNAs (296, 297) together with emerging technologies (298) and collaboration with computational methods (299, 300) will aid in delineating the cell-specific roles of these transcripts that are not only conserved at the sequence level, but also at the secondary structure level (301). But with no high-throughput methodology yet to assess lincRNA function, there exists a need to first identify and then filter lincRNAs in a cell-specific context. Identifying lincRNAs that display evolutionary sequence conservation across species in a particular tissue provides a first step in this endeavor.

4.2 Rationale and methodology to reveal evolutionary conservation of lincRNAs across species as a determinant of functional preservation in the eye

4.2.1 Rationale for research approach

Discovery of wide-spread non-coding RNAs such as lincRNAs in the mammalian genome has challenged our understanding of the cellular transcriptional/translational landscape. Recently lincRNAs have been increasingly associated with heritable human disorders, especially neurodevelopmental disabilities. The dynamic process of vision mediated by neuronal cells of the retina can potentially be mediated by lincRNAs. But learning which lincRNAs are important in tissue specific contexts is difficult because as of yet there is no high-throughput methodology to assess lincRNA function. Examining adult species with fully developed retinas with different behavior patterning and
photoreceptor populations can identify those lincRNAs that persisted in sequence conservation and are thus indicative of potentially preserved function in the face of evolution.

Using high-throughput sequencing allows one to focus on lincRNAs that could coordinate essential retinal transcriptional homeostatic functions by identifying those that were evolutionarily conserved in the eye and retina across a diverse range of mammals, including humans. An integrative experimental and bioinformatic set of approaches can further show lincRNA expression profiles restricted to specific retinal layers. Examination of the genomic loci of the lincRNAs and analysis of their promoter regions can also provide a functional basis for those conserved lincRNAs in regulating adult retinal function. Probing the transcription factor binding sites can further reveal if these lincRNAs contain promoter elements for integral retinal transcription factors. Together these may provide evidence that lincRNAs are not only are required for homeostasis, but also could serve as potentially novel therapeutics for retinopathies.

4.2.2 Materials and methods

**Eye and retina tissue collection.** A/J, BALB/c, C57BL/6 (B6), and Cone–DTA strains of mice, all 4 weeks of age, were obtained from The Jackson Laboratory (Bar Harbor, ME). *P23H* knock–in mice generated in our laboratory (302) were 7 months of age. Four–week–old *Nrl*–deficient mice with a B6 background were obtained from Dr. Anand Swaroop (University of Michigan, Ann Arbor, MI). Long–Evans rats (*Rattus norvegeicus*), 4 weeks of age, were purchased from Harlan Laboratories (Madison, WI). Nile rats (*Arvicanthis niloticus*), 6 weeks of age, were obtained from the laboratory of Dr. Laura Smale (Michigan State University, Lansing, MI). Wild caught adult ground
squirrels (*Ictidomys tridecemlineatus*) were purchased from TLS Research (Bloomingdale, IL). Mice, rats and ground squirrels were housed in the CWRU animal facility where they were maintained on a standard chow diet in a 12 h light (~10 lux)/12 h dark cycle. After euthanizing animals, eye and retina tissue were collected and placed in a solution of RNAlater (Qiagen) for processing. Enucleated macaque (*Macaca fascicularis*) eyes in RNALater from 4–year-old animals were obtained from Ricerca Biosciences (Painesville, OH). Clinical evaluations of the human patient from whom retinal tissue was obtained were carried out at the Cleveland Clinic Cole Eye Institute (Cleveland, OH). This research conformed to the tenets of the Declaration of Helsinki. The retina was carefully dissected out of an untreated eye from a patient requiring enucleation for a large ocular melanoma and immediately placed in RNALater; the retinal sample was obtained from a hemi–retina free of tumor. This eye had no abnormal neovascularization of the iris or retina and lacked signs of inflammation.

**Library preparation and Illumina RNA–Seq runs.** Eye and retina tissue libraries were prepared as described in preceding chapters. Each mouse library was run on the Illumina Genome Analyzer IIx in the CWRU Genomics core facility using 36– to 79–base pair single–end read lengths. The processed and raw fastq files were previously deposited in GEO (accession numbers GSE38359 and GSE29752). Prepared libraries of Long–Evans rat eye and retinal tissues and human retinal tissue were sequenced by single–end sequencing whereas prepared libraries of Nile rat, ground squirrel, and macaque eye and retina tissue were sequenced by paired–end sequencing technology with the Illumina Genome Analyzer IIx or HiScan SQ.
Expression of lincRNAs in mouse eye and retina. Sequence information of 3133 identified lincRNAs in the mouse was extracted from Ensembl release 67. The Illumina reads from each mouse replicated tissue sample were processed separately. A quality trimming step was performed to remove bases from both ends with quality scores equivalent to a Phred quality score lower than 20. Only reads with 20 bases or longer after quality trimming were retained for further analysis. Trimmed reads were aligned to the mouse genome release mm9 with TopHat v2.0.0 (303). Aligned reads were assembled under the guidance of mm9 RefSeq and Ensembl lincRNA transcripts. Expression values were calculated as fragments per kilobase of exon model per million mapped reads (FPKM) for each lincRNA.

Conservation of mouse lincRNAs in different species. Reads from eye and retinal tissue samples of the different species were trimmed of bases with Phred quality scores of lower than 20. Reads that were less than 20 bases after such trimming were discarded. Reads from technical replicate lanes were combined before being mapped to the mouse lincRNA sequences with TopHat v2.0.0 (303) and the resulting alignment files were processed with custom Perl scripts. Alignments with large (>3 base) insertions or deletions or reads of low complexity (≥80% of the reads were di–nucleotide repeats or contained 8 or more consecutive A– or T–bases) were removed before the number of original reads and unique reads aligned to mouse lincRNA transcripts were counted. Portions of mouse lincRNA transcripts covered by reads were calculated and presented as bases and portions of the entire length of the transcript. If a mouse lincRNA transcript was aligned to 5 or fewer unique reads from a sample or the read coverage of the
lincRNA transcript was less than 10%, then the mouse lincRNA transcript was not considered to be conserved in that species sample.

**Promoter analysis.** Each 5 kilobase promoter sequence of the 18 conserved lincRNA transcripts was searched with FIMO (304) for motifs in the JASPAR CORE 2009 database ([http://jaspar.genereg.net/](http://jaspar.genereg.net/)) and only those that returned statistically significant P values with a position–specific scoring matrix for each of the motifs in the promoter sequences were considered. The 5 kilobase promoter sequences of all annotated genes in mouse genome release mm9 were also searched for motifs in the JASPAR CORE 2009 database. Motif enrichment in the promoter regions of the 18 conserved lincRNAs compared to genome–wide promoter regions was assessed with the Fisher's exact test using a Bonferroni correction.

**Immunohistochemistry.** All procedures used were reported previously (228). Cross–sections of mouse eyecups were incubated with primary antibodies, namely anti–mouse RPE65, biotinylated peanut agglutinin (PNA) and wheat germ agglutinin (WGA). Signals were detected with Cy3– or Alexa488–conjugated secondary antibody. Nuclear staining was achieved with 4’,6–diamidino–2–phenylindole (DAPI). Sections were analyzed with a Leica TCS SP5 II confocal microscope (Leica, Wetzlar, Germany).

**In situ hybridization.** The QuantiGene ViewRNA (Affymetrix, Santa Clara, CA) *in situ* hybridization protocol was optimized by Affymetrix for use with fresh frozen mouse eye tissue samples. The protease concentration was increased four–fold from a 1:100 to a 1:25 dilution and the incubation time was set at 40 minutes from the standard protocol for optimized signal strength and tissue morphology preservation. Slides were incubated with
EyeLinc2 (VB1–13468), EyeLinc7/8 (VB1–13469), EyeLinc14 (VB1–13470) and EyeLinc17 (VB1–13471) Fast Red probe sets for detection with a Leica TCS SP5 II confocal microscope. Slides incubated with the mouse Ubc (VB1–10202) Fast Red probe set were used for a positive control whereas slides incubated with the E. coli dapB (VF1–10272) Fast Red probe set, employed as a negative control, were used to establish a background signal for the assay. Rat kidney FFPE tissue slides as a positive (Rat Ubc probe set, VC1–10190) and a negative (E. coli dapB probe set, VF1–10272) control were also analyzed to demonstrate that the assay reagents and the assay protocol were properly followed.

**Semi–quantitative real–time PCR.** Total RNA from B6 mouse tissues, including eye, brain, heart, liver, lung, cornea/lens, retina and RPE/choroid was purified by using the RNeasy Mini Kit with on column DNase treatment (Qiagen, Valencia, CA). Each of the 18 lincRNAs identified to be conserved in all species was probed with the Qiagen One–step RT–PCR Kit. Twenty five ng of total RNA was used in each 12.5 μL reaction as per the manufacturer’s instructions. Primers used to probe lincRNAs were custom designed to span introns whenever possible to rule out genomic DNA contamination. Actin primers were designed for loading controls and primers against Nrl (retina) and Rpe65 (RPE) were employed to confirm the fidelity of tissue dissections.

**Quantitative real–time PCR.** One μg of isolated eye, brain, heart, liver and lung tissue RNA from 2 pooled B6 mice, 1 μg of cornea/lens, retina, and RPE/choroid tissue RNA from 2 pooled B6 mice and 1 μg of eye tissue RNA from B6, Cone–DTA, Nrl−/−, and P23H mice were converted to cDNA with the High Capacity RNA–to–cDNA kit (Applied Biosystems, Foster City, CA). RT–PCR was done with TaqMan chemistry and
Assays on Demand probes (Applied BioSystems) for mouse Abca4 (Mm00492035_m1), Opn1sw (Mm00432058_m1), Rpe65 (Mm00504133_m1), and ABI custom designed primers for EyeLinc1 (AIX00TR), EyeLinc2 (AIW2NJ), EyeLinc4 (AIS074V), EyeLinc7/8 (AIVI4HB), EyeLinc14 (AIT96A3), and EyeLinc17 (AIRR9YN). The 18S rRNA (4319413E) probe set (Applied BioSystems) was used as the endogenous control.

All real-time experiments were done in triplicate with the ABI StepOne Plus qRT-PCR machine (Applied BioSystems). Fold changes were calculated based on differences in threshold cycles (Ct) after normalization to 18S rRNA. Percent relative expression is presented as a percent of the maximal normalized expression observed in the different samples.

**Statistical Analysis.** Experimental results were analyzed by an independent two-sample t-test. A P value of 0.05 or less was considered statistically significant. Data presented graphically in figures are shown as means ± standard deviations.

### 4.3 Results

4.3.1 RNA-Seq identifies eye lincRNAs that exhibit sequence conservation in mammals and those that exhibit conservation in the human retina and macular region

RNA-Seq was carried out with eye tissue from a diverse set of mammalian species with varied retinal photoreceptor cellular compositions and distributions. First, 3 biological replicates of whole eye tissue from mice with 3 distinct genetic backgrounds: A/J, BALB/c, and C57BL/6J (B6) were analyzed. An RNA-Seq experiment of a retinal sample from B6 mice was also included to guide transcript localization. Expression
values were calculated as fragments per kilobase per million reads (FPKM) (133). With an expression cut–off of 1 FPKM, 103, 83, and 109 lincRNAs were detected in A/J, BALB/c and B6 mouse eyes, respectively, of which 48 were consistently expressed (Figure 41A). These 48 lincRNAs were then investigated for conservation at the sequence level in eye tissue from three rodents with a range of photoreceptor populations and behavior patterning, namely Rattus norvegeicus (rat, nocturnal, ~1% cones), Arvicanthis niloticus (Nile rat, diurnal, ~33% cones), and Ictidomys tridecemlineatus (ground squirrel, diurnal, ~97% cones), as well as the higher order mammal, Macaca fasicularis (monkey, diurnal, 5% cones, macula present). Data were analyzed by quality trimming the reads from each species and mapping them to the 48 consistently expressed mouse lincRNA sequences.

Using this methodology, 18 mouse lincRNAs were found to be conserved in the eye across all species examined (Figure 41A-B). Many of these lincRNAs exhibited extremely high sequence conservation and further examination revealed that 16 of the 18 lincRNAs were also conserved in human retinal tissue subjected to RNA–Seq analysis (Figure 41C). The conservation profile of lincRNAs in the eye and retina of the species (Tables 8-9) indicated that most localized to the retina, a perception further supported by examining the expression profile in B6 mice (Table 10). Although high order mammals like monkeys and humans have only about 5% cone photoreceptors in their retina (305), a central region of the retina termed the macula, most specifically the fovea, is rich in cone photoreceptors and responsible for visual acuity and high–resolution vision (306). By specifically sequencing the macular region from 4 monkey biological replicates we found that 14 of the 18 conserved lincRNAs across species were present (Figure 42, Table 11).
Next, these 18 transcripts were investigated in greater detail to determine their tissue-specific expression profiles in B6 mice.

4.3.2 Tissue and eye compartment expression of conserved lincRNAs

Expression profiles of the 18 conserved lincRNAs were investigated by isolating total RNA from a range of tissues in B6 mice and subjecting them to RT-PCR analysis. Eye, brain, heart, liver and lung tissue were chosen for their cellular complexity and diversity of biological function. Eye compartments including the lens/cornea, retina, and RPE/choroid were also examined separately. Specific primers used to amplify the product sequence of lincRNAs were subjected to Sanger sequencing which revealed 100% sequence overlap with the annotated sequence in Ensembl. Semi-quantitative RT–PCR (sqRT–PCR), which was used to determine transcript presence and absence at optimized cycles, revealed the tissue enrichment profile of these lincRNAs (Table 12). These results were further evaluated with quantitative RT–PCR (qRT–PCR) of 6 selected lincRNAs that displayed broad organ and eye tissue distribution in expression profiles (Figure 43). The sqRT–PCR and qRT–PCR results were in good agreement, with subtle differences attributable to the use of different custom primer sets for the experiments. A subset of lincRNAs evidenced expression restricted to just a few organs and specific eye compartments. EyeLinc14, for example, was more highly expressed in the eye relative to other organs examined and localized preferentially to the retina and RPE/choroid compartments. Targets such as EyeLinc1 and EyeLinc4 showed enrichment in just a few tissues whereas others like EyeLinc17 were expressed in most tissues assayed. Although many lincRNAs showed preferential expression in the retinal compartment of the eye, it was then necessary to determine if they were localized to specific retinal cell types.
4.3.3 Expression is localized to specific retinal layers of some conserved lincRNAs

To localize lincRNAs in the retina, first mouse models were utilized with different rod/cone ratios, a methodology previously employed to localize microRNAs to cellular components of the retina (307). Whereas in B6 mice all cellular components of the retina were preserved, in \(Cone^{-DTA^{+/-}}\) mice \((Cone^{-DTA})\) cone photoreceptors were absent but rod photoreceptors were preserved (308), in \(NrL^{-/-}\) mice rod photoreceptors were absent and only cone–like photoreceptors were present (53), and in aged (7–month–old) \(P23H\) mice there were no photoreceptors detected but other layers of the neural retina were retained (302) (Figure 44A). Total RNA extracted from eyes of these mice was then subjected to RT–PCR analysis for lincRNA expression levels. The sqRT–PCR (Table 13) results, further quantified by qRT–PCR of selected targets (Figure 44B), suggested possible localization of the lincRNAs to specific retinal layers. Enriched expression of \(EyeLinc2\) and \(EyeLinc7/8\) in B6 and \(Cone^{-DTA}\) mice together with greatly reduced expression in \(NrL^{-/-}\) mice and some residual expression in \(P23H\) mice suggested their possible localization to the photoreceptor layer, as this pattern resembled the profile of \(Abca4\) expression, with a gene product that resides in photoreceptor discs (309). \(EyeLinc14\) displayed higher expression in B6 and \(Cone^{-DTA}\) mice compared with reduced expression in \(NrL^{-/-}\) and \(P23H\) mice. Markedly reduced \(Eyelinc14\) expression in the latter 2 mouse models suggests that this lincRNA primarily localizes to both rod photoreceptors and other retinal cellular compartments such as the RPE, in agreement with the results shown in Figure 43.
LincRNA localization inferred from these mouse models, was further investigated by carrying out in situ hybridization of EyeLinc2, EyeLinc7/8, EyeLinc14, and EyeLinc17 in the mouse retina by establishing a new methodology in frozen mouse retinal sections that allowed sensitive single–molecule RNA detection with almost no background. Mouse ubiquitin c (Ubc) was used as a positive control whereas E. coli dihydrodipicolinate reductase (dapB) was used as a negative control for these hybridization assays. All lincRNAs were detected above the negative control background and signals in the photoreceptor regions were localized to the inner photoreceptor cell segments (Figure 44C). EyeLinc14 was notably detected in both the photoreceptor inner segment and RPE layers, consistent with localization findings from previous PCR experiments shown in Figures 43 and 44B. Therefore, the localization of these lincRNAs to specific retinal compartments, especially their enrichment in the neuronal photoreceptor cell layer, suggested that lincRNAs could have an important role in supporting vision in adult retinas. To further substantiate this, computational analysis of the conserved lincRNA loci and analysis of their promoter regions for binding motifs of transcription factors were carried out.

4.3.4 Genetic loci and in silico analyses of promoter motifs highlight possible roles of lincRNAs in adult retinal homeostasis

EyeLinc2 not only lies adjacent to the photoreceptor gene Abca4 in the mouse genome (Figure 45A), but its expression pattern is suggestive of photoreceptor localization (Figure 44B-C). Support for possible involvement of these lincRNAs in retinal homoeostasis was investigated by analyzing transcription factor–binding motifs present in the 5–kilobase promoter regions of all 18 conserved lincRNAs with the
JASPAR CORE 2009 database. Promoter motifs for binding of 17 unique transcription factors were found in these 18 lincRNAs, of which 13 mapped to multiple transcripts (Table 14). Analysis of transcription factor–binding site motifs in the context of retinal cell biology suggested that these conserved lincRNAs could play physiological roles in retinal homeostasis. Investigation of the promoter region of EyeLinc2 revealed binding sites for genes such as \textit{HMG-I(Y)} (p=2.13×10^{-6}) and \textit{Sp1} (p=6.58×10^{-6}) implicated in regulating photoreceptor gene expression (310, 311) (Figure 45A). Consistent with the retina (312) and long non–coding RNAs (313) displaying circadian rhythmicity, \textit{EyeLinc2} displayed statistically significant temporal changes in its expression level during the day (Figure 45A). The promoter regions of \textit{EyeLinc7/8}, \textit{EyeLinc14}, and \textit{EyeLinc17} further illustrate binding motifs of other genes implicated in photoreceptor and adult retinal homeostasis (Figure 45B) such as \textit{Pax4} (314) and \textit{CTCF} (315, 316) of which \textit{CTCF} has been shown to regulate gene expression in concert with a non–coding RNA (317).

4.4 Discussion and conclusions

The hypothesis that long non–coding RNAs in intergenic regions of the human genome possess functional roles (318) in normal homeostasis of post–mitotic retinal neurons and that their disregulation can lead to human diseases (319) necessitates a detailed understanding of these transcripts. The sheer number of the lincRNA population makes it difficult to elucidate their physiological contribution to a specific tissue, but a first step would be to identify those that are conserved across species in specific tissues. Using high–throughput RNA–Seq methodology 18 lincRNAs were identified in the adult
eye that showed sequence conservation across a diverse range of species, from nocturnal rod–dominant rodents such as mouse and rat, to cone–dominant diurnal rodents such as the Nile rat and ground squirrel, to the diurnal monkey which like humans, possesses a cone–rich macula and rod–rich peripheral retina. Moreover, 16 of these 18 lincRNAs were found conserved in the human retina with 14 of these 18 also conserved in the macular region of the retina. A subset of these lincRNAs exhibited expression restricted to certain tissues in the mouse. Mouse models with different retinal cell populations along with in situ hybridization were used to further localize these lincRNAs to specific cellular layers of the retina, most notably the neuronal photoreceptor layer. Complementation of the cellular work with computational analysis of transcription factor–binding site motifs of the 18 conserved lincRNAs was a critical step in revealing a contribution of these transcripts in retinal homeostasis. In the absence of proper retinal cell lines to accurately identify in vivo functionality of these transcripts, more detailed roles of individual lincRNAs should become evident from cellular phenotypes that result when the cohort of conserved lincRNA loci identified in these experiments are disrupted.

Evolutionary pressure drives rapid sequence alterations of lincRNAs even in closely related species (320) highlighting the functional importance of those transcripts that remain conserved across species (321). Identification of 18 conserved lincRNAs from the initial 3133 mouse lincRNAs agrees with past studies that only a small minority of lincRNAs in mouse or human have transcribed orthologous sequences in the other species (286). Identification of conserved lincRNAs that persisted despite evolutionary pressure across the diverse adult species studied (Figures 41 and 42) implies that these transcripts could potentially coordinate essential transcriptional/translational roles in
adults when all retinal components are fully developed. These conserved lincRNAs exhibited a 100–fold expression range in the B6 mouse eye and retina (Table 10), but even those marginally expressed lincRNAs could fulfill vital role such as basal regulation of protein–coding genes (319).

Closer examination of these 18 lincRNAs in B6 mice revealed that whereas some transcripts like EyeLinc17 displayed ubiquitous expression in the several tissues assayed, others like EyeLinc14 displayed tissue–restricted expression. EyeLinc14 not only displayed enriched expression in the eye relative to other organs assayed, but it also was preferentially located in the retina and RPE, two layers that intimately collaborate to drive vision. Expression in tissues outside the eye does not preclude those lincRNAs from having retina–specific roles. Indeed Tug1, a non–coding RNA shown to be important at early stages of photoreceptor development, displayed robust expression in tissues besides the eye (277). As to the retina, lincRNAs were first localized in mouse models with subtle differences in retinal architecture (Figure 44A-B) that had been previously used to localize non–coding RNAs (307). In situ hybridization (Figure 44C) was then used to localize certain lincRNAs to specific retinal compartments, most notably to the neuronal photoreceptor and RPE layers of the retina.

Complementation of the above cellular studies with computational analysis of the genomic loci and promoter regions of all conserved lincRNAs provided insights into their possible roles in retinal homeostasis (Figure 45). EyeLinc2 exhibited an enrichment and localization pattern suggestive of photoreceptor expression (Figure 44B-C) and lies adjacent in the mouse genome to Abca4 (Figure 45A), an essential photoreceptor protein product for removal of toxic retinoid metabolites (322) that can cause severe
retinopathies if mutated (323, 324). Promoter analysis of EyeLinc2 revealed that it possesses binding sites for \( HMG-I(Y) \), which is rarely expressed in terminally differentiated cells, but it is enriched in photoreceptor cells and thought to accommodate the daily induction of phototransduction and visual cycle genes such as \( Abca4 \) (311). Consistent with the diurnal nature of gene expression in the retina, HMG–box proteins evidence diurnal rhythms in photoreceptors (325), and EyeLinc2 also displayed temporal changes in expression levels with a 1.7–fold elevation in the afternoon as compared to the morning (Figure 45A). Temporal cycling of lincRNAs in the eye adds another dimension to their possible regulatory function (313). Examination of other lincRNAs revealed promoter regions for genes such as \( Pax4 \) and \( CTCF \). Unlike the master regulator homeobox–containing \( Pax6 \) involved in eye morphogenesis (326), both \( Pax4 \) (314) and \( CTCF \) (316) are developmentally segregated and display highest expression in photoreceptor cells of the adult retina. \( Pax4 \) is thought to regulate gene expression in the mature retina (314) and can activate expression of rod–derived cone viability factor (327), a novel trophic factor that can protect cone photoreceptors from degenerating (328) and thus serve as alternative therapy for patients with retinitis pigmentosa (329). Meanwhile, recent evidence revealed that \( CTCF \) regulates the ataxin–7 gene through interactions with a non–coding RNA (317). Dysfunction in ataxin–7 leads to spinocerebellar ataxia type 7 and has been shown to produce photoreceptor dysfunction (330, 331) and retinal degeneration (332). Moreover, overlapping disease pathways recently demonstrated for this neurodegenerative disorder and age–related macular degeneration (333) highlight that these lincRNAs can have homeostatic roles beyond the retina. Thus, the in silico analysis highlights potential involvements of these conserved
lincRNAs, not only in the maintenance of adult retinal homeostasis, but also in interacting with key retinal transcription factors for potential therapy of human retinopathies.

The dynamic process of vision requires a high level of expression to maintain photoreceptor specific genes that carry out phototransduction and the visual cycle (281), a process potentially affected by lincRNAs (334). This study suggests how in adult terminally differentiated post–mitotic retinal photoreceptor cells, lincRNAs could play a critical role in physiology of these cells. As understanding of the transcriptional landscape in human cells improves (335), accurate assembly of lincRNAs in tissue-specific contexts through high-throughput sequencing approaches constitutes the first step to identifying those transcripts that are likely to be important and warrant more detailed investigation. With increasing evidence that long non–coding RNAs such as lincRNAs are associated with common diseases (336) and constitute potential drivers of cancerous states (337), it is imperative to investigate those that could influence such pathology. That these transcripts can be targeted in mouse models of human disease to correct pathological states (338) also provides hope that their improved understanding will shape future therapeutics.
Table 8. Profile of 18 conserved lincRNAs in the eye across species. Data presented in each cell represent the total number of reads (after trimming) aligned to the listed transcript; the unique number of reads (after trimming) aligned to the transcript; the length of the transcript covered by the aligned reads; and the percentage of the transcript covered by the aligned reads. Thus for *EyeLinc1* in the rat eye, there were 206 total reads and 166 unique reads. The unique reads covered 1578 bases of the transcript, representing a 65% conservation of *EyeLinc1*.

<table>
<thead>
<tr>
<th>lincRNA</th>
<th>Rat Eye</th>
<th>Nile Rat Eye</th>
<th>Ground Squirrel Eye</th>
<th>Monkey Eye 1</th>
<th>Monkey Eye 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>EyeLinc1</td>
<td>206; 166; 1578; 0.65</td>
<td>1400; 1147; 1781; 0.73</td>
<td>1402; 707; 920; 0.38</td>
<td>732; 404; 889; 0.37</td>
<td>910; 448; 851; 0.35</td>
</tr>
<tr>
<td>EyeLinc2</td>
<td>22644; 1412; 926; 0.51</td>
<td>92086; 14333; 960; 0.53</td>
<td>5470; 1794; 720; 0.40</td>
<td>8077; 911; 573; 0.32</td>
<td>7471; 896; 644; 0.36</td>
</tr>
<tr>
<td>EyeLinc3</td>
<td>318; 243; 1458; 0.46</td>
<td>1087; 914; 1610; 0.50</td>
<td>88; 50; 507; 0.16</td>
<td>134; 86; 498; 0.16</td>
<td>98; 66; 441; 0.14</td>
</tr>
<tr>
<td>EyeLinc4</td>
<td>2938; 404; 426; 0.62</td>
<td>3667; 1800; 451; 0.66</td>
<td>147; 137; 305; 0.45</td>
<td>127; 124; 288; 0.42</td>
<td>165; 154; 336; 0.49</td>
</tr>
<tr>
<td>EyeLinc5</td>
<td>13067; 966; 652; 0.95</td>
<td>27310; 7132; 686; 1.00</td>
<td>1956; 740; 556; 0.81</td>
<td>293; 273; 437; 0.64</td>
<td>310; 291; 512; 0.75</td>
</tr>
<tr>
<td>EyeLinc6</td>
<td>3685; 1370; 1980; 0.87</td>
<td>3391; 2760; 2023; 0.88</td>
<td>14; 14; 251; 0.11</td>
<td>100; 83; 307; 0.13</td>
<td>63; 52; 298; 0.13</td>
</tr>
<tr>
<td>EyeLinc7</td>
<td>20691; 1390; 1308; 0.59</td>
<td>8713; 4384; 1438; 0.65</td>
<td>1026; 881; 1065; 0.48</td>
<td>673; 595; 968; 0.44</td>
<td>526; 471; 1050; 0.48</td>
</tr>
<tr>
<td>EyeLinc8</td>
<td>20802; 1394; 1337; 0.61</td>
<td>8459; 4279; 1394; 0.63</td>
<td>1053; 917; 1052; 0.48</td>
<td>673; 606; 975; 0.44</td>
<td>487; 443; 1062; 0.48</td>
</tr>
<tr>
<td>EyeLinc9</td>
<td>364; 135; 288; 0.47</td>
<td>509; 348; 337; 0.55</td>
<td>19; 18; 158; 0.26</td>
<td>7; 7; 90; 0.15</td>
<td>7; 7; 126; 0.20</td>
</tr>
<tr>
<td>EyeLinc10</td>
<td>199; 138; 512; 0.72</td>
<td>1050; 705; 642; 0.90</td>
<td>62; 32; 279; 0.39</td>
<td>41; 26; 197; 0.28</td>
<td>120; 45; 243; 0.34</td>
</tr>
<tr>
<td>EyeLinc11</td>
<td>2308; 250; 443; 0.66</td>
<td>7850; 2682; 480; 0.72</td>
<td>101; 96; 379; 0.57</td>
<td>65; 64; 295; 0.44</td>
<td>79; 76; 290; 0.43</td>
</tr>
<tr>
<td>EyeLinc12</td>
<td>1344; 747; 2128; 0.64</td>
<td>1086; 816; 1663; 0.50</td>
<td>16; 16; 361; 0.11</td>
<td>82; 60; 366; 0.11</td>
<td>138; 82; 365; 0.11</td>
</tr>
<tr>
<td>EyeLinc13</td>
<td>1718; 498; 697; 0.82</td>
<td>5607; 2486; 812; 0.96</td>
<td>3354; 434; 652; 0.77</td>
<td>2519; 356; 626; 0.74</td>
<td>3625; 422; 621; 0.73</td>
</tr>
<tr>
<td>EyeLinc14</td>
<td>8210; 949; 731; 0.29</td>
<td>66474; 10987; 971; 0.38</td>
<td>17614; 1643; 629; 0.25</td>
<td>10515; 778; 574; 0.23</td>
<td>14392; 871; 586; 0.23</td>
</tr>
<tr>
<td>EyeLinc15</td>
<td>1086; 729; 1973; 0.67</td>
<td>1649; 972; 1984; 0.68</td>
<td>658; 307; 910; 0.31</td>
<td>882; 356; 753; 0.26</td>
<td>1369; 419; 733; 0.25</td>
</tr>
<tr>
<td>EyeLinc16</td>
<td>328; 210; 830; 0.44</td>
<td>1125; 986; 979; 0.51</td>
<td>37; 36; 304; 0.16</td>
<td>35; 35; 354; 0.19</td>
<td>36; 36; 298; 0.16</td>
</tr>
<tr>
<td>EyeLinc17</td>
<td>40701; 2842; 1968; 0.97</td>
<td>89765; 21461; 2023; 1.00</td>
<td>2673; 1005; 1490; 0.74</td>
<td>620; 585; 1266; 0.62</td>
<td>830; 783; 1412; 0.70</td>
</tr>
<tr>
<td>EyeLinc18</td>
<td>5922; 650; 581; 0.54</td>
<td>11471; 3716; 585; 0.55</td>
<td>1089; 541; 489; 0.46</td>
<td>299; 281; 476; 0.44</td>
<td>364; 349; 544; 0.51</td>
</tr>
</tbody>
</table>
Table 9. Profile of 18 conserved lincRNAs in the retina across species. Data presented in each cell represent the total number of reads (after trimming) aligned to the listed transcript; the unique number of reads (after trimming) aligned to the transcript; the length of the transcript covered by the aligned reads; and the percentage of the transcript covered by the aligned reads. Thus for *EyeLinc1* in the rat eye, there were 593 total reads and 466 unique reads. The unique reads covered 1634 bases of the transcript, representing a 67% conservation of *EyeLinc1*. Although conserved in the eye, *EyeLinc6*, *EyeLinc9*, and *EyeLinc16* appear not to be conserved in the retina across species.

<table>
<thead>
<tr>
<th></th>
<th>Rat Retina</th>
<th>Ground Squirrel Retina</th>
<th>Monkey Retina 1</th>
<th>Monkey Retina 2</th>
<th>Human Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EyeLinc1</em></td>
<td>593; 466; 1634; 0.67</td>
<td>4798; 1744; 1003; 0.41</td>
<td>621; 370; 848; 0.35</td>
<td>777; 406; 840; 0.35</td>
<td>536; 256; 841; 0.35</td>
</tr>
<tr>
<td><em>EyeLinc2</em></td>
<td>19251; 1604; 1035; 0.57</td>
<td>10557; 3959; 735; 0.41</td>
<td>5500; 844; 571; 0.32</td>
<td>5228; 540; 597; 0.33</td>
<td>214; 154; 707; 0.39</td>
</tr>
<tr>
<td><em>EyeLinc3</em></td>
<td>945; 605; 1612; 0.50</td>
<td>226; 179; 887; 0.28</td>
<td>147; 83; 454; 0.14</td>
<td>134; 66; 537; 0.17</td>
<td>19; 12; 355; 0.11</td>
</tr>
<tr>
<td><em>EyeLinc4</em></td>
<td>2152; 430; 453; 0.66</td>
<td>255; 229; 260; 0.38</td>
<td>135; 127; 244; 0.36</td>
<td>80; 77; 351; 0.51</td>
<td>55; 43; 334; 0.49</td>
</tr>
<tr>
<td><em>EyeLinc5</em></td>
<td>11455; 1132; 647; 0.94</td>
<td>2046; 1179; 462; 0.67</td>
<td>321; 293; 446; 0.65</td>
<td>195; 182; 520; 0.76</td>
<td>210; 177; 617; 0.90</td>
</tr>
<tr>
<td><em>EyeLinc6</em></td>
<td>18; 17; 457; 0.20</td>
<td>0</td>
<td>32; 28; 237; 0.10</td>
<td>0</td>
<td>7; 7; 229; 0.10</td>
</tr>
<tr>
<td><em>EyeLinc7</em></td>
<td>14840; 1491; 1341; 0.61</td>
<td>2103; 1551; 1017; 0.46</td>
<td>1341; 1080; 1026; 0.47</td>
<td>401; 369; 885; 0.40</td>
<td>127; 122; 620; 0.28</td>
</tr>
<tr>
<td><em>EyeLinc8</em></td>
<td>14845; 1481; 1340; 0.61</td>
<td>2219; 1651; 1051; 0.48</td>
<td>1329; 1082; 972; 0.44</td>
<td>401; 374; 799; 0.36</td>
<td>134; 130; 564; 0.26</td>
</tr>
<tr>
<td><em>EyeLinc9</em></td>
<td>310; 151; 290; 0.47</td>
<td>49; 47; 189; 0.31</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>EyeLinc10</em></td>
<td>174; 135; 513; 0.72</td>
<td>74; 41; 362; 0.51</td>
<td>51; 30; 170; 0.24</td>
<td>53; 31; 240; 0.34</td>
<td>7; 6; 173; 0.24</td>
</tr>
<tr>
<td><em>EyeLinc11</em></td>
<td>1843; 294; 477; 0.71</td>
<td>191; 180; 334; 0.50</td>
<td>55; 54; 249; 0.37</td>
<td>40; 40; 315; 0.47</td>
<td>22; 19; 278; 0.42</td>
</tr>
<tr>
<td><em>EyeLinc12</em></td>
<td>1133; 705; 2001; 0.60</td>
<td>38; 37; 492; 0.15</td>
<td>98; 65; 374; 0.11</td>
<td>121; 70; 357; 0.11</td>
<td>0</td>
</tr>
<tr>
<td><em>EyeLinc13</em></td>
<td>1963; 515; 724; 0.85</td>
<td>4165; 890; 662; 0.78</td>
<td>1343; 279; 636; 0.75</td>
<td>1885; 291; 576; 0.68</td>
<td>281; 117; 651; 0.77</td>
</tr>
<tr>
<td><em>EyeLinc14</em></td>
<td>7716; 995; 767; 0.30</td>
<td>20731; 2599; 726; 0.28</td>
<td>6189; 637; 558; 0.22</td>
<td>8126; 587; 577; 0.23</td>
<td>787; 187; 638; 0.25</td>
</tr>
<tr>
<td><em>EyeLinc15</em></td>
<td>1922; 1107; 2005; 0.69</td>
<td>592; 335; 998; 0.34</td>
<td>959; 380; 809; 0.28</td>
<td>994; 348; 748; 0.26</td>
<td>74; 49; 856; 0.29</td>
</tr>
<tr>
<td><em>EyeLinc16</em></td>
<td>761; 421; 1108; 0.58</td>
<td>81; 76; 328; 0.17</td>
<td>28; 28; 220; 0.12</td>
<td>0</td>
<td>28; 27; 192; 0.10</td>
</tr>
<tr>
<td><em>EyeLinc17</em></td>
<td>55865; 3937; 1978; 0.98</td>
<td>7086; 3757; 1597; 0.79</td>
<td>554; 536; 1278; 0.63</td>
<td>380; 370; 1187; 0.59</td>
<td>237; 168; 1720; 0.85</td>
</tr>
<tr>
<td><em>EyeLinc18</em></td>
<td>4086; 650; 594; 0.55</td>
<td>1381; 913; 520; 0.48</td>
<td>390; 367; 451; 0.42</td>
<td>212; 206; 483; 0.45</td>
<td>133; 106; 523; 0.49</td>
</tr>
</tbody>
</table>
Table 10. Transcript abundance (FPKM) in the eye and retina of B6 mice for the 18 lincRNAs conserved across species and protein coding photoreceptor and RPE genes *Abca4*, *Opn1mw*, and *Rpe65*. Similarities in the abundance of many of these lincRNAs between whole eye and retina indicate their probable localization.

<table>
<thead>
<tr>
<th></th>
<th>B6 Eye</th>
<th>B6 Retina</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EyeLinc1</strong></td>
<td>4.31</td>
<td>5.16</td>
</tr>
<tr>
<td><strong>EyeLinc2</strong></td>
<td>35.22</td>
<td>38.05</td>
</tr>
<tr>
<td><strong>EyeLinc3</strong></td>
<td>2.58</td>
<td>3.25</td>
</tr>
<tr>
<td><strong>EyeLinc4</strong></td>
<td>85.89</td>
<td>39.22</td>
</tr>
<tr>
<td><strong>EyeLinc5</strong></td>
<td>108.25</td>
<td>73.18</td>
</tr>
<tr>
<td><strong>EyeLinc6</strong></td>
<td>6.62</td>
<td>0.14</td>
</tr>
<tr>
<td><strong>EyeLinc7</strong></td>
<td>4.94</td>
<td>5.64</td>
</tr>
<tr>
<td><strong>EyeLinc8</strong></td>
<td>4.94</td>
<td>5.64</td>
</tr>
<tr>
<td><strong>EyeLinc9</strong></td>
<td>1.73</td>
<td>1.18</td>
</tr>
<tr>
<td><strong>EyeLinc10</strong></td>
<td>4.74</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>EyeLinc11</strong></td>
<td>16.07</td>
<td>3.29</td>
</tr>
<tr>
<td><strong>EyeLinc12</strong></td>
<td>7.72</td>
<td>11.28</td>
</tr>
<tr>
<td><strong>EyeLinc13</strong></td>
<td>11.93</td>
<td>4.33</td>
</tr>
<tr>
<td><strong>EyeLinc14</strong></td>
<td>7.51</td>
<td>10.12</td>
</tr>
<tr>
<td><strong>EyeLinc15</strong></td>
<td>4.46</td>
<td>4.86</td>
</tr>
<tr>
<td><strong>EyeLinc16</strong></td>
<td>9.15</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>EyeLinc17</strong></td>
<td>199.39</td>
<td>193.64</td>
</tr>
<tr>
<td><strong>EyeLinc18</strong></td>
<td>30.57</td>
<td>21.36</td>
</tr>
<tr>
<td><strong>Abca4</strong></td>
<td>59.00</td>
<td>140.14</td>
</tr>
<tr>
<td><strong>Opn1mw</strong></td>
<td>117.79</td>
<td>187.21</td>
</tr>
<tr>
<td><strong>Rpe65</strong></td>
<td>67.21</td>
<td>17.63</td>
</tr>
</tbody>
</table>
Table 11. Profile of 18 conserved lincRNAs in 4 biological replicates of macaque macula tissue. Data presented in each cell represent the total number of reads (after trimming) aligned to the listed transcript; the unique number of reads (after trimming) aligned to the transcript; the length of the transcript covered by the aligned reads; and the percentage of the transcript covered by the aligned reads. In the monkey retina *EyeLinc6, EyeLinc9* (both not conserved in the human retina), and *EyeLinc16* are not conserved, and in the macula in addition to these three, *EyeLinc11* also is not conserved.

<table>
<thead>
<tr>
<th></th>
<th>Monkey Macula 1</th>
<th>Monkey Macula 2</th>
<th>Monkey Macula 3</th>
<th>Monkey Macula 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>EyeLinc1</em></td>
<td>333; 235; 936; 0.39</td>
<td>1162; 487; 969; 0.40</td>
<td>793; 368; 954; 0.39</td>
<td>660; 354; 954; 0.39</td>
</tr>
<tr>
<td><em>EyeLinc2</em></td>
<td>4014; 139; 361; 0.20</td>
<td>13510; 165; 426; 0.24</td>
<td>11480; 164; 388; 0.22</td>
<td>14567; 158; 397; 0.22</td>
</tr>
<tr>
<td><em>EyeLinc3</em></td>
<td>178; 120; 523; 0.16</td>
<td>380; 155; 575; 0.18</td>
<td>306; 125; 535; 0.17</td>
<td>428; 163; 527; 0.16</td>
</tr>
<tr>
<td><em>EyeLinc4</em></td>
<td>25; 17; 117; 0.17</td>
<td>51; 26; 115; 0.17</td>
<td>88; 31; 116; 0.17</td>
<td>80; 32; 212; 0.31</td>
</tr>
<tr>
<td><em>EyeLinc5</em></td>
<td>329; 32; 183; 0.27</td>
<td>1440; 68; 353; 0.51</td>
<td>1494; 56; 305; 0.44</td>
<td>1069; 46; 304; 0.44</td>
</tr>
<tr>
<td><em>EyeLinc6</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>EyeLinc7</em></td>
<td>1251; 38; 343; 0.16</td>
<td>2301; 37; 248; 0.11</td>
<td>2833; 42; 267; 0.12</td>
<td>2775; 44; 312; 0.14</td>
</tr>
<tr>
<td><em>EyeLinc8</em></td>
<td>1251; 38; 343; 0.16</td>
<td>2301; 37; 248; 0.11</td>
<td>2833; 42; 267; 0.12</td>
<td>2775; 44; 312; 0.14</td>
</tr>
<tr>
<td><em>EyeLinc9</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>EyeLinc10</em></td>
<td>23; 21; 169; 0.24</td>
<td>117; 69; 174; 0.24</td>
<td>64; 41; 170; 0.24</td>
<td>65; 51; 176; 0.25</td>
</tr>
<tr>
<td><em>EyeLinc11</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>EyeLinc12</em></td>
<td>94; 68; 382; 0.11</td>
<td>173; 101; 514; 0.15</td>
<td>183; 73; 392; 0.12</td>
<td>134; 82; 384; 0.12</td>
</tr>
<tr>
<td><em>EyeLinc13</em></td>
<td>1124; 247; 557; 0.66</td>
<td>3821; 268; 560; 0.66</td>
<td>2962; 264; 557; 0.66</td>
<td>2772; 263; 561; 0.66</td>
</tr>
<tr>
<td><em>EyeLinc14</em></td>
<td>3189; 320; 498; 0.20</td>
<td>9711; 332; 499; 0.20</td>
<td>8754; 337; 498; 0.20</td>
<td>7520; 323; 500; 0.20</td>
</tr>
<tr>
<td><em>EyeLinc15</em></td>
<td>306; 186; 544; 0.19</td>
<td>1022; 326; 635; 0.22</td>
<td>787; 285; 598; 0.20</td>
<td>676; 261; 598; 0.20</td>
</tr>
<tr>
<td><em>EyeLinc16</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>EyeLinc17</em></td>
<td>3521; 99; 398; 0.20</td>
<td>9897; 110; 577; 0.28</td>
<td>7958; 112; 630; 0.31</td>
<td>9995; 108; 630; 0.31</td>
</tr>
<tr>
<td><em>EyeLinc18</em></td>
<td>268; 58; 240; 0.22</td>
<td>638; 79; 302; 0.28</td>
<td>801; 77; 292; 0.27</td>
<td>779; 73; 271; 0.25</td>
</tr>
</tbody>
</table>
Table 12. Semi-quantitative RT-PCR of conserved lincRNAs in organs and eye compartments of 1 month old C57BL/6 mice. Experiments were optimized for expression of each listed transcript in the eye. Bands detected at the established optimized cycle in other tissues are denoted with a “+”; a “−” indicates that no band was detected. For three targets, namely EyeLinc9, EyeLinc12 and EyeLinc18, we failed to detect expression in any tissues, even after using two different sets of custom designed primers. ND signifies ‘not detected’.

<table>
<thead>
<tr>
<th></th>
<th>Organs</th>
<th>Eye Compartments</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Eye</td>
<td>Brain</td>
</tr>
<tr>
<td><strong>EyeLinc1</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>EyeLinc2</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc3</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc4</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc5</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc6</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>EyeLinc7</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>EyeLinc8</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>EyeLinc9</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>EyeLinc10</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc11</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc12</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td><strong>EyeLinc13</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc14</strong></td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><strong>EyeLinc15</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc16</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc17</strong></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>EyeLinc18</strong></td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>
Table 13. Semi-quantitative RT-PCR of conserved lincRNAs in mice with different retinal environments. Experiments were optimized for the expression of each transcript in the B6 eye. Bands detected at this established optimized cycle in eyes of mice with other genotypes are denoted with a “+”. A “-” indicates that no band was detected.

<table>
<thead>
<tr>
<th></th>
<th>B6 Eye</th>
<th>ConeDTA Eye</th>
<th>Nrl⁻⁻⁻ Eye</th>
<th>P23H Eye</th>
</tr>
</thead>
<tbody>
<tr>
<td>EyeLinc1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc4</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EyeLinc5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EyeLinc8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EyeLinc10</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc11</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EyeLinc13</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc15</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc16</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>EyeLinc17</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table 14. *In silico* promoter analysis of conserved lincRNAs reveals binding sites for transcription factors that drive retinal processes. Results of motif searches that returned statistically significant P values (p ≤ 0.05) for each 5k promoter sequence of the 18 conserved lincRNA transcripts are shown after analysis with the JASPAR CORE 2009 database. Numbers in parentheses next to each promoter motif indicate the number of occurrences of that motif.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>EyeLinc1</strong></td>
<td>EWSR1-FLII(1), Foxd3(1), RREB1(9), RTG3(1), SP1(1)</td>
<td>EWSR1-FLII(1), HMG-I/Y(2), RREB1(8), SP1(2), Trl(1)</td>
<td>CTCF(2), PPARG::RXRA(1), Pax4(1), RREB1(6), SP1(13), Trl(1)</td>
<td>CTCF(2), EWSR1-FLII(2), FKH1(1), RREB1(13), SP1(1), squamosa(1)</td>
<td>EWSR1-FLII(1), HMG-I/Y(1), RREB1(2), Trl(3)</td>
<td>CTCF(2), EWSR1-FLII(1), PHD1(1), PLAG1(1), RREB1(41), SP1(12), Trl(1)</td>
<td>EWSR1-FLII(1), FKH1(1), Foxd3(1), HMG-I/Y(3), Pax4(1), RREB1(25), SP1(3), Trl(41), fkh(2)</td>
<td>EWSR1-FLII(1), FKH1(1), Foxd3(1), HMG-I/Y(3), Pax4(1), RREB1(27), SP1(3), Trl(44), fkh(2)</td>
<td>FKH1(1), Pax4(1), RREB1(24), SP1(10)</td>
<td>EWSR1-FLII(2), SP1(1)</td>
<td>CTCF(1), EWSR1-FLII(36), RREB1(9), SP1(8), Trl(6)</td>
<td>EWSR1-FLII(2), RREB1(4), SP1(1)</td>
<td>EWSR1-FLII(18), Foxd3(1), NHP6B(2), RREB1(19), SP1(14), Trl(19)</td>
<td>CTCF(1), EWSR1-FLII(3), RREB1(25), SP1(4)</td>
<td>EWSR1-FLII(2), FKH1(1), Foxd3(2), HMG-I/Y(1), PHD1(1), PPARG::RXRA(1), Pax4(3), RREB1(29), SP1(4), Trl(1)</td>
<td>CTCF(1), EWSR1-FLII(2), HMG-I/Y(1), PHD1(1), RREB1(5), SP1(6)</td>
<td>NHP6B(1), PPARG::RXRA(1), Pax4(1), RREB1(3), SP1(5)</td>
<td>EWSR1-FLII(4), HMG-I/Y(1), Pax4(1), RREB1(8), SP1(1), nub(1)</td>
</tr>
</tbody>
</table>
Figure 41. RNA−Seq of eye tissue from rodents and higher order mammals reveals sequence conserved lincRNAs. (A) RNA−Seq of three biological replicates of whole eye tissues from A/J, BALB/c, and C57BL/6J mice revealed 103, 83, and 109 lincRNAs (≥1 FPKM) in A/J, BALB/c and C57BL/6 mouse eyes, respectively, of which 48 were found consistently expressed across the three different mouse backgrounds. Of these 48 lincRNAs, 18 showed sequence conservation across different rodent species with variable retinal photoreceptor cell populations (Rattus norvegicus (rat), nocturnal, ~1% cones; Arvicanthis niloticus (Nile rat), diurnal, ~33% cones; and Ictidomys tridecemlineatus (ground squirrel), diurnal, ~97% cones) as well as a higher order mammal (Macaca fasicularis (monkey), diurnal, 5% cones, macula present). (B) Diagram illustrating the conservation profile of the 48 mouse expressed lincRNAs with 18 conserved across all species and 5 conserved exclusively among rodents. Of the 18 conserved eye lincRNAs, 16 also were conserved in human retinal tissue. (C) Shown is an example of reads mapped to a mouse lincRNA. ENSMUST00000170823 was chosen as it was the most highly expressed of the 18 conserved mouse lincRNAs. Portions of this mouse lincRNA transcript covered by reads from eye tissue of different species are presented. Interestingly, reads for the second exon were found in rodents closer to mouse (Rattus norvegicus and Arvicanthis niloticus), but not detected in Ictidomys tridecemlineatus, Macaca fasicularis or Homo sapiens samples. A table of all 18 conserved lincRNAs is shown below. For each species the lincRNA transcript covered by reads was calculated and presented as the total number of nucleotides (nt) in the specified lincRNA transcript and portions of the entire length of the transcript covered by the reads. For example, of the 2,247 bases of EyeLinc1 in Rattus norvegicus there were reads of 1,578 bases
encompassing 0.65 of the entire transcript. The 16 lincRNAs listed in blue also exhibited sequence conservation in the human retina.
Figure 42. LincRNAs displaying conservation in the macular region of the retina highlight their potential role in higher order visual processing. RNA–Seq analysis of 4 biological replicates of monkey macula tissue revealed that of the 18 conserved lincRNAs identified across all species, 14 of them (shown in blue) were conserved in the macular region.
Figure 43. LncRNAs display spatially restricted expression in adult B6 mouse organs and eye compartments. Semi-quantitative RT–PCR of all 18 conserved lincRNAs revealed tissue specific expression, such as for EyeLinc14, which not only revealed enriched expression in the eye relative to other organs but was also more preferentially expressed in the retina and RPE/choroid. Rpe65, a gene expressed only in the RPE of the eye, is shown as a positive control whereas Actin was used for a loading control. The heat map below shows qRT–PCR results for 6 selected lincRNAs and 2 positive controls (Opn1sw and Rpe65) in the different organs and eye compartments. EyeLinc7 and EyeLinc8, identical in sequence from a duplication in the X chromosome, are listed as EyeLinc7/8. Areas are shaded according to the relative levels of transcription normalized to the tissue/compartment with the highest expression levels ranging from 0% (white, undetectable) to 100% (black, highest).
Figure 44. Enrichment profiles in mouse models with varying photoreceptor populations and in situ hybridization reveal cellular localization of lincRNAs to specific retinal layers. Mouse models that differ in their retinal architecture were analyzed: (A) B6 mice exhibited a fully intact retina with staining of the RPE layer as well as rod and cone photoreceptors; Cone−DTA mice featured an intact retina with RPE and rod photoreceptor staining but no cone photoreceptor signals; Nrl−/− mice displayed staining of the RPE layer and cone photoreceptors but no rod photoreceptor signals; and aged P23H mice had a degenerated photoreceptor layer with severely attenuated residual rod and cone photoreceptor staining, whereas the RPE layer stained with an antibody against RPE65 was normal. Rods and cones were stained with the lectins, WGA and PNA, respectively. Nuclei were stained with DAPI. (B) Expression of selected lincRNAs assessed by qRT−PCR of eye tissue from these mouse models is shown as a heatmap shaded as in Figure 43. In addition to the 6 selected lincRNAs, 3 controls were run: Abca4, a gene product that resides in photoreceptor discs; Opn1sw, a cone photoreceptor marker; and Rpe65, a RPE cell marker. (C) Mouse Ubc and E. coli dapB were used as positive and negative controls, respectively, to carry out in situ hybridizations in mouse retinas. Nuclei were stained with DAPI. LincRNA probes exhibiting the most prominent signals in the photoreceptor regions were localized to inner segments (denoted by downward−pointing white arrows), with expression in some cases in the RPE layer (denoted by upward−pointing white arrows). Layers are labeled as RPE, retinal pigmented epithelium; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
**Figure 45.** Location in the mouse genome and promoter analysis highlight possible roles for lincRNAs in retinal homeostasis. (A) Chromosomal location of the *Abca4* and *Bcar3* genes and the intergenic *EyeLinc2*. Plots show the RNA–Seq read coverage of these transcripts in the whole eye and retina of B6 mice. The promoter regions relative to the transcribed region of *EyeLinc2* highlight conservation of each promoter motif and the corresponding sequence found along with the P value for each motif occurrence are displayed. *EyeLinc2* also exhibited temporal changes in expression with a 1.7-fold increased expression at 9 h compared to 1.5 h after lights were turned on (p<0.01). (B) Promoter regions relative to transcribed regions highlight different transcription factor–binding motifs found in *EyeLinc7/8, EyeLinc14* and *EyeLinc17*. 
The genotype to phenotype phenomenon is complex conundrum to decipher. High-throughput sequencing technology has provided more complete and unbiased views of the whole genome and tissue specific transcriptomes. Furthermore, recent innovations in functional and structural characterization of tissues and organs have allowed better assignment of phenotypes associated with certain traits or disease. In simple model organisms such as the budding yeast *Saccharomyces cerevisiae*, careful phenotypic and genotypic dissection have revealed the complex set of background-specific modifiers that exist. These factors make it difficult to identify the genetic basis for individual phenotypes, but studying such model organisms provides the framework to understand the genetic networks that underlie human physiology and disease (339). Model organisms offer a more complete and systematic way to study and connect genes and phenotypes to provide a more unbiased assessment of the genetic complexity of phenotypic traits (340). The elucidation of the genetic architecture of these traits can be directly related to human physiology and disease as genes work in evolutionary conserved pathways.

The goal of this work was to understand the phenotypic and genotypic factors that contribute to normal physiological function of the retina and those changes that lead to disease. Visual loss in individuals can have profound personal, economic, and social implications and with the predictions by the National Eye Institute that the incidence of blindness will double in 20 years, effective preventive measures must be developed for these diseases, which can only be established as more is known about their etiology (341). By interfacing high-resolution hybrid microscopy techniques with high-throughput RNA-Seq methodology, this work provided the genotype to phenotype connection in both rare Mendelian and more common complex multi-genic retinal dystrophies.
Moreover, the work unveiled that adult retinal function may be under an additional level of transcriptional regulation by lincRNAs.

In Mendelian diseases, causative genes can be identified, but these diseases are rare in the population, making it difficult to study. Even in cases where the causative gene has been elucidated, the pathophysiology of the disease may still remain unclear. An excellent example of this is in enhanced S-cone syndrome. Although researchers identified the causative mutations decades ago that result in the developmental overproduction of S-cones in the retina, the failure to explain the progressive degenerative component of the disease limited the knowledge necessary for design of proper therapeutics. In this work, by directly comparing data from a cohort of human ESCS patients, it was shown that knockout of a key developmental transcription factor, Nrl, in mice resulted in the same phenotypic features of the human disease. Since disruption of the normal retinal development patterning leads to an overproduction in S-cones it was hypothesized that there would be homoeostatic disruption in the retina, most specifically the photoreceptor layer, accounting for the progressive degeneration. RNA-Seq of normal and Nrl knockout mice carried out an early age before degeneration was present revealed that the ESCS mice had decreased expression of key photoreceptor genes that promote disc shedding and subsequent RPE-mediated phagocytosis. Phenotypic characterization of ESCS photoreceptors using hybrid FIB-SEM methodology revealed that there was abnormal accumulation of membranes in the photoreceptor outer segment at the RPE interface indicative of a failure to properly shed disc membranes. Moreover, examination of the RPE-photoreceptor interface with SBF-SEM imaging, supported the defect in proper phagocytosis as that there was an
attenuation of phagocytotic events in the RPE in ESCS but not normal mice. The most important conclusion from the mouse studies was that these phenotypic features directly correlated with findings from human patients. There had been indications from non-invasive retinal imaging modalities that there was some disruption at the RPE-photoreceptor interface in ESCS patients, but the small sample size did not allow for any definitive conclusions. By studying the disease more systematically using a mouse model of the disease, this work revealed that overcoming the retinal photoreceptor shedding and phagocytosis defect could potentially arrest the progressive degenerative component of ESCS.

As human life expectancy has increased, so has the incidence of complex age-related diseases. In humans, AMD is one of the major blinding diseases worldwide, but the paucity of effective treatment options stems from the poorly understood complex disease etiology. This complex multi-genic etiology makes it difficult to find an appropriate animal model to study. However, phenotypic variability based on genetic backgrounds in highly inbred strains of mice provide models for such a complex human condition. Inbred strains of mice were thus investigated and it was shown that of the three background strains, A/J, BALB/c, and B6, A/J displayed the most pronounced features of ARD whereas B6 had no age-related phenotype and BALB/c showed a less severe ARD phenotype than A/J mice. A more exhaustive temporal phenotypic characterization of these mice revealed that prior to noticeable visual function decline in A/J mice, pathological features were evident in the RPE layer that became exacerbated with increasing age and translated to photoreceptor pathology. Comprehensive genetic studies utilizing CSS and RNA-Seq of young mice before the visual decline revealed aberrant
gene networks provide inadequate protection by the RPE from age-related stress, which drives the retina from an inflammatory primed state to a chronic disease state in A/J mice. This work highlighted how subtle genetic differences can have profound effect on the predisposition to and pathogenesis of ARD. Furthermore, this work also provided new insight into the human condition. Not only were aberrant gene networks correlated with previous genome wide association studies results, but it was shown that the additive effects of the inflammation and the deterioration of the RPE/retina barrier can lead to secondary effects of the disease such as autoimmune responses to retinal antigens. It was also noted that in ARD mice, the post-mitotic photoreceptor and RPE cells underwent detrimental cellular senescence at an early age. Recent developments that clearance of such senescent cells can not only delay age-related pathology, but delay already established age-related disease (223), offer a new therapeutic option for this complex retinal disease. Interestingly, it had been noted from genome wide associate studies that the vast majority of variations related to complex trait analysis fall in non-coding regions of the genome (274). Re-examination of the RNA-Seq data of the different background mice revealed that a proportion of the transcript reads resided in intergenic regions of the genome and mapped to known mouse lincRNA transcripts. This prompted study of these non-coding transcripts to understand if they are involved in retinal homeostasis.

The majority of transcription gives rise to non-coding RNAs rather than protein-coding transcripts, and as organism complexity rises, so does non-coding RNA content compared to protein-coding transcripts. Thus, phenotypic variation between individuals or species may be based largely on differences in non-coding RNA sequences (342). Examining the lincRNA profile across the different inbred species with differing rates of
ARD revealed 74 lincRNAs with varying expression profiles, some uniquely higher in one background whereas some were higher in shared backgrounds. The phenotypic complexity in ARD may thus be explained by the differential profile of these lincRNAs, but with no annotated function except a few such transcripts, it was first necessary to elucidate if lincRNAs may even mediate normal retinal physiology. To identify such lincRNAs, an evolutionary approach was taken. Evolutionary pressure has been shown to drive rapid sequence alternations in even closely related species, therefore, those that display evolutionary sequence conservation across species would be indicative of functional importance. RNA-Seq of eye tissues from a diverse set of mammalian species with varied retinal photoreceptor cellular compositions and distributions, in addition to the inbred mouse lines, revealed sequence conservation of 18 mouse lincRNAs. Furthermore, 16 of these 18 lincRNAs were also conserved in the human retina and 14 of the 18 were conserved in the macular region of monkeys. Of these 18 transcripts, some displayed ubiquitous tissue expression in B6 mice. On the other hand some displayed very specific tissue expression, with one lincRNA found exclusively enriched in the eye and specifically to the retina and RPE compartments of the eye. A functional role of these transcripts in the eye was inferred from transcription factor-binding site motifs in the context of retinal cell biology. Many of the promoter motifs encoding transcription factors were not only uniquely expressed in the retina, but were specifically localized to the photoreceptors. Moreover, a subset of these eye lincRNAs displayed oscillating expression, highlighting that it may be involved in critical circadian processes in the eye such as diurnal photoreceptor phagocytosis (343). Most importantly, this work provided the first evidence that a cohort of lincRNAs that may be critical for retinal physiology in
adults. That these identified lincRNAs display promoter motifs of proteins that can be disrupted in retinal disease and that these transcripts can be targeted in mouse models of human disease to correct pathological states \( (338) \) opens a new door for potential transcriptional regulation in the retina and therapeutic options moving forward.

This work highlights how interfacing high-resolution imaging modalities with RNA-Seq technology allows one to connect the phenotype to genotype from rare Mendelian disease to more common, yet complex multi-genic diseases. Moreover, the elucidation of lincRNAs provides a new model of transcriptional control that may mediate normal and disease processes in the retina. Future work will look to extend this approach to examine basic physiologic processes in the retina that have eluded our complete understanding. One such process is the circadian shedding of photoreceptor discs and subsequent RPE-mediated phagocytosis and processing. This process is essential for removal of toxic metabolites and lifelong survival of the post-mitotic photoreceptors. The severe retinal degenerative phenotypes that arise from genetic defects in critical components of this RPE mediated process, such as the Mer tyrosine kinase (MerTK) \( (344, 345) \), further highlight the importance of this circadian process. Much of our knowledge regarding RPE-mediated phagocytosis has been guided by the mechanism utilized by professional macrophages, but the biochemical processes such as the role of second messengers in RPE mediated phagocytosis is still poorly defined. The circadian clock network contributes to physiological responses by intersecting with cell-specific transcripts \( (346) \). Therefore, transcriptome analysis of the oscillating transcripts in the eye can provide insight of how genomic rhythms are transformed into metabolic physiology in the eye. Analysis of lincRNAs in the B6 mouse eye have already showed
that some displaying oscillating expression, highlighting that this process may also be mediated by both protein-coding and non-coding transcripts. To connect the outward phenotype to the underlying genetic drivers of this circadian process will require elucidating of the phenotypic features and combining that with temporal genetic information. SBF-SEM has already been used to quantify phagocytosis in normal and ESCS mice. Since this serial sectioning methodology can be used to quantify phagocytosis events across large portions of the retina, if B6 mice are taken shortly after light onset coinciding with the peak of phagocytosis events, and then hours later in the afternoon during the trough of phagocytosis events, the difference can be accurately quantified. With the establishment of the temporal phenotype, global gene expression can be carried out at these designated time points with RNA-Seq. The use of three biological replicates at each time point will then allow for identification of those transcripts that display statistically significant oscillations in expression. Both protein-coding and lincRNA transcripts will be analyzed. Moreover, the essential circadian clock genes will be investigated to observe if they display oscillating expression as in other cell types (347). For those oscillating transcripts, it will then be necessary to ascertain if these proteins are expressed in the RPE cell layer, and thus could modulate cellular physiology associated with photoreceptor phagocytosis. This localization will be assessed by immunohistochemical staining. As the core clock genes dictate the expression of effector genes that drive circadian physiology, the promoter region of such effector oscillating transcripts will be investigated to identify if they contain binding sites of such transcription factors. Finally, recent studies from circadian cistromic studies of the liver (348) will be used to see if Bmal1 and Rev-erbα/β, components of the positive and
negative limbs of the circadian clock machinery, respectively, co-occupy and thus control the temporal expression of the oscillating transcripts identified in the eye. The goal of this future work will be to highlight the intricacies that exist in the genomic architecture of the eye that serve to tightly control the circadian process of photoreceptor phagocytosis and maintain the health of post-mitotic photoreceptor over the lifetime of the organism.

Another future direction of this work will be to better elucidate the genetic modifiers that exist to modulate disease in different backgrounds. Identified disease causing mutations are not always fully penetrant, and a possible reason for this is that outcome of this mutation depends on other genetic variations that exist in the genome. This dependence of mutation outcome on genetic background is referred to as epistasis (349). This idea was evident in the work regarding ARD, where the chromosome substitution strains exhibited no ARD phenotype. Epistasis and its interactions have been implicated in human disease. Moreover, epistatic interactions have led to over estimations of the heritability in human disease (350). Model organisms can be used to systematically study and understand epistatic interactions in greater detail and better predict disease outcome. Even in Mendelian diseases like ESCS, epistasis may be a central determinant of pathology, and perhaps the severity of the progressive degenerative component of the disease as evidenced by varying rates of degeneration in ESCS diseased mice on differing backgrounds (351). By better understanding the epistasis in the context of ARD, a more complete estimation of heritability can be inferred for this disease. This can be carried out by again using the A/J, BALB/c, and B6 inbred mouse lines and generating crosses of each strain against the others. Given the chromosome substitution strain experiments, one would expect the A/J x B6 cross to be
resistant to ARD, but the real interesting point will be if there is some rescue of the phenotype in the A/J x BALB/c cross, which would highlight the dominance of BALB/c and B6 strains and the epistatic nature of genetic interactions driving ARD. The expression of select genes in F1 progeny from these initial crosses can be analyzed one month after birth as even at that early point, genetic signatures of ARD have been identified. If there is a degree of gene expression rescue to non-symptomatic B6 levels, the mice can be followed to see if the development of ARD is retarded, or if further F2 crosses back to the dominant genetic background have an even more pronounced rescue of the genotype and phenotype.

The final future aspect of this work would be to delineate the functional role of lincRNAs in the retina. The evolutionary sequence conservation search and subsequent biochemical and bioinformatic analyses provided possible roles of these transcripts in mediating retinal physiology, but the definitive roles of individual lincRNAs should become evident from cellular phenotypes that result when the cohort of conserved lincRNA loci identified in these experiments are disrupted. The lack of proper retinal cell line in which to properly knock down and study the effects lincRNAs would have on retinal function necessitates that genomic disruption in vivo by creating knockout mice. In conventional gene-targeting methods, a gene knockout is introduced through homologous recombination in mouse embryonic stem cells, which are then targeted into wild type blastocysts to generate chimeric animals to generate mice with the targeted knockout. This not only a costly endeavor, but also very time intensive taking between 6 to 12 months. The recent discovery of transcription activator-like effector nucleases (TALENs) has overcome many of the limitations of embryonic stem cell based
conventional gene targeting by inducing mutations at specific genomic loci (352). Moreover, TALENs have been used to create gene knockout mice (353). Another emerging technology is the type II bacterial clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) systems that has been demonstrated to not only to be efficient at gene-targeting but also has the potential for multiplexed genome editing, all of which can be carried out in a single step (354). These new methodologies will facilitate the study of the lincRNAs in the retina by not only allowing quicker delivery of knockout mice, but also for the analysis of the phenotypic effects when multiple lincRNAs are knocked out. Given that lincRNA mouse model knockouts have not shown a robust phenotype to date and the idea that lincRNAs may have overlapping functions, this approach will allow for the identification of those lincRNAs that either singly or in concert, mediate retinal homeostasis in the adult eye.
REFERENCES


