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Genetics And Retinal Degeneration: Challenges In Optogenetic Therapy And Indentifying Pathogenic Variants

Abstract
Inherited retinal degenerative diseases are one of the leading causes of childhood blindness. While over 200 causative genes have been identified, many cases still have an unknown underlying genetic cause. With the advent of next generation sequencing (NGS), it should be possible to identify the genetic cause in almost every case, provided enough relatives are willing to participate. However, the massive amount of data generated by NGS can make identifying the pathogenic variant challenging. It is necessary to filter the data in order to create a manageable candidate list, but overly strict filtering or erroneous assumptions can result in filtering out the pathogenic variant. Identifying the genetic cause of retinal degeneration in each patient will allow us to better identify candidate genes for gene therapy and bring us a step closer to precision medicine. Here we developed an efficient screening system to find candidate mutations with minimal assumptions to avoid screening out pathogenic variants and better identify good candidates for novel gene discovery. Out of an initial cohort of 69 patients we identify the pathogenic variant(s) in 44 of them and identified 11 subjects as good candidates for novel gene discovery.

We also need a broad treatment for retinal degeneration to help those who have mutations in genes that are poor candidates for gene therapy or who have an unknown genetic cause. We tested the efficacy of using the GRM6 minimal promoter as a bipolar cell specific promoter to express channelrhodopsin in bipolar cells after photoreceptor degeneration to make the bipolar cells directly respond to light. Surprisingly, we found that unlike in the wildtype mouse retina, the GRM6 promoter is not bipolar cell specific in multiple mouse models of retinal degeneration. This suggests that the genetic profiles of the cells in the inner retina change during retinal degeneration. Understanding these fundamental changes in cell specific gene expression during retinal degenerative processes will be critical in order to develop effective therapeutic strategies for late stage retinal degeneration.

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GENETICS AND RETINAL DEGENERATION: CHALLENGES IN OPTOGENETIC THERAPY AND INDENTIFYING PATHOGENIC VARIANTS

Laura M. Bryant

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ABSTRACT

GENES AND RETINAL DEGENERATION: CHALLENGES IN OPTOGENETICS THERAPY AND IDENTIFYING PATHOGENIC VARIANTS

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Jean Bennett M.D., Ph.D.

Inherited retinal degenerative diseases are one of the leading causes of childhood blindness. While over 200 causative genes have been identified, many cases still have an unknown underlying genetic cause. With the advent of next generation sequencing (NGS), it should be possible to identify the genetic cause in almost every case, provided enough relatives are willing to participate. However, the massive amount of data generated by NGS can make identifying the pathogenic variant challenging. It is necessary to filter the data in order to create a manageable candidate list, but overly strict filtering or erroneous assumptions can result in filtering out the pathogenic variant. Identifying the genetic cause of retinal degeneration in each patient will allow us to better identify candidate genes for gene therapy and bring us a step closer to precision medicine. Here we developed an efficient screening system to find candidate mutations with minimal assumptions to avoid screening out pathogenic variants and better identify good candidates for novel gene discovery. Out of an initial cohort of 69 patients we identify the pathogenic variant(s) in 44 of them and identified 11 subjects as good candidates for novel gene discovery.

We also need a broad treatment for retinal degeneration to help those who have mutations in genes that are poor candidates for gene therapy or who have an unknown genetic cause. We tested the efficacy of using the *GRM6* minimal promoter as a bipolar cell specific promoter to express channelrhodopsin in bipolar cells after photoreceptor degeneration to make the bipolar cells directly respond to light. Surprisingly, we found that unlike in the wildtype mouse retina, the *GRM6* promoter is not bipolar cell specific in multiple mouse models of retinal degeneration. This suggests that the genetic profiles of the cells in the inner retina change during retinal degeneration. Understanding these fundamental changes in cell specific gene expression during retinal degenerative processes will be critical in order to develop effective therapeutic strategies for late stage retinal degeneration.

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Chapter 1: Introduction

Phenotype and genotype variability in inherited retinal degeneration

Hereditary retinal degeneration causes blindness independent of environmental factors, with disease onset often occurring during childhood. This includes non-syndromic forms like Stargardt disease, Leber's congenital amaurosis (LCA), retinitis pigmentosa (RP), achromatopsia and cone-rod dystrophy (CORD) as well as syndromic forms like Bardet-Biedl syndrome (BBS), Usher's syndrome and Batten disease. Typically, hereditary retinal degeneration is monogenic and has an earlier onset than other causes of retinal degeneration like glaucoma or age related macular degeneration. In some forms, such as LCA, disease onset is very young, before the age of 5[1]. Others, like RP and Stargardt can have an adult onset[2,3]. Retinal dystrophy can be inherited in every known inheritance pattern including autosomal dominant (sometimes with incomplete penetrance), autosomal recessive, X-linked and sporadic. There is a huge variability in disease presentation and progression. Stargardt disease primarily affects cones while RP primarily affects rods[4,5]. More severe diseases like LCA start by affecting rods but eventually cause secondary cone degeneration[6]. Other forms, such as cone dystrophy, largely preserve rod function, even in the most advanced disease state[7]. Some forms of retinal degeneration cause different patterns of degeneration or include deposits in the subretinal space. The hallmark feature of Stargardt disease is yellow flecks in the macula, which are lipofuscin deposits in the subretinal space[3,4,8]. This is not seen in other forms of retinal degeneration. Meanwhile, bone spicules, caused by changes in the

retinal pigment epithelial (RPE) cells after photoreceptor degeneration, are characteristic of retinitis pigmentosa[9].

Despite the huge variability seen in different forms of retinal degeneration, there is also significant overlap in disease phenotype which can affect the ability to give an accurate and specific diagnosis in early stages of degeneration. In particular, syndromic retinal degeneration can appear to be non-syndromic in early stages if other symptoms have not yet appeared or if patients fail to report the other symptoms to the ophthalmologist. Bardet Biedl syndrome is extremely variable in severity and mild forms can be initially diagnosed as RP[10]. Cone dystrophy can progress to cone rod dystrophy in some cases while in other cases rods are spared[11]. Since the only difference is disease progression, an ophthalmic exam during the early stages of the diseases cannot always provide an exact diagnosis. When the diagnosis is being made based on the time of disease onset and severity of degeneration, it is possible that a genetic diagnosis can modify the initial clinical diagnosis, such as changing an initial diagnosis of RP to a late onset form of LCA or an initial diagnosis of BBS can be changed to RP after clinical reassessment[12,13].

In addition to the phenotypic variability in retinal degeneration, there is significant genetic variability within the same phenotype. There are currently 18 genes known to cause LCA, with a significant number of patients with no known genetic cause[14-18]. Retinitis Pigmentosa can be caused by mutations in any one of over 60 genes, with approximately 40% of cases negative for all known causes of RP[19,20]. Other forms have less genetic variability. Stargardt disease, for example, is almost always caused by mutations in *ABCA4* (95% of cases)[21]. Many of the genes that cause retinal degeneration

can cause more than one form. For example, although *ABCA4* causes most cases of Stargardt disease, it can also lead to retinitis pigmentosa or cone-rod dystrophy or even act as a disease modifying allele[22-24]. The same mutation in *PRPH2* can cause autosomal dominant RP or macular degeneration even within the same family[25]. Variants in *USH2A* have been shown to cause both Usher syndrome and RP[26]. These are only a few of many examples of genes that can cause more than one phenotype.

Possibilities for polygenic inheritance

To further complicate the genetics of retinal degeneration, digenic inheritance is another possible mode of inheritance. So far only one digenic cause of retinal degeneration has been identified (*ROM1* and *PRPH2*), but it is likely that at least some of the remaining cases of undetermined genetic cause will be digenic or polygenic[27]. Recently, a second potential cause of digenic retinal degeneration (this time causing syndromic retinal dystrophy) was proposed that is caused by null alleles in *RP1L1* and *C2orf71*, but more has to be done to conclusively prove that heterozygous mutations in these two genes are sufficient to cause retinal degeneration in humans[28].

While there is so far only one known cause of digenic inheritance, modifying alleles are much more common and contribute to the phenotypic variability we see with some genes. Heterozygous mutations that would not cause retinal degeneration by themselves have been shown to contribute to disease pathogenesis when combined with a pathogenic mutation, either alleviating or aggravating the disease phenotype. *ABCA4* and *ROM1* can act as modifying alleles for *PRPH2* mutations and determine rod vs cone phenotype[24].

RPGRIP1L can act as a modifying allele in ciliopathies to exacerbate (or cause) a retinal phenotype[29]. While not pathogenic in isolation, these mutations are very important to note and can help provide a more accurate clinical diagnosis and prediction of disease progression. They can be particularly important in cases of autosomal dominant inheritance with incomplete penetrance. In those cases, modifying alleles can prevent development of the disease in individuals carrying the pathogenic variant. For example, variants in *PRPF31* can cause autosomal dominant retinitis pigmentosa through hemizygous insufficiency[30]. However, a variant in *CNOT3* upregulates the expression of *PRPF31*, thereby increasing the expression of the wildtype allele to a level that is sufficient to meet the needs of the cell. Family members with the

How different cell types contribute to retinal degeneration

Typically mutations that cause retinal degeneration are in genes expressed in the photoreceptors or the retinal pigment epithelium (RPE), although not necessarily exclusively expressed in those cell types. Photoreceptor degeneration is often the first stage of retinal degeneration, so it follows that mutations in photoreceptor genes can directly affect photoreceptor viability. The RPE provides structural and trophic support to the photoreceptors, and mutations there can also have a direct impact on photoreceptor viability. Rarely, mutations in genes in the choroid, like *CA4*, can also cause retinal degeneration through a more complicated mechanism.

Photoreceptors are uniquely susceptible to degeneration due to their high metabolic load, high protein turnover and unique structure[32-34]. Each photoreceptor has an outer segment

containing stacks of membrane (called discs) and is connected to the cell body by the connecting cilium[35]. Each disc contains the proteins needed for phototransduction[36]. All of the proteins in the outer segment must be trafficked through the connecting cilium[37]. Any deficit in cilia trafficking (such as those caused by mutations in *RPGR, ALMS1* or *ARL3*) can have a profound detrimental effect on the outer segments. The structure of the outer segment ensures that almost every particle of light will hit a disc and induce the phototransduction cascade but it also makes the cell particularly dependent on the primary cilia. Mutations in genes responsible for cilia structure and maintenance (such as *CEP29* and, *MAK*) or in disc structure (like *PRPH2* and *ROM1*) can cause retinal degeneration by destabilizing the outer segment or prevent the outer segment from forming properly[38]. Mutations in proteins involved in the phototransduction cascade (such as *RHO, SAG* and *PDE6B*) prevent proper regulation of the ion channels involved in phototransduction[39]. Excess or deficiency in ions, especially calcium, can induce apoptosis[40]. Mutations in transcription factors like *CRX* and *NR2E3* result in photoreceptor degeneration by impairing and altering development, thereby preventing proper photoreceptor formation[41,42]. Meanwhile, mutations in splicing factors (which include *PFPF3*, *PRPF8* and *PRPF31*) appear to affect photoreceptor viability more indirectly[43]. It is still unknown why mutations in ubiquitously expressed splicing factors, which are required by every cell type, can cause tissue specific deficits. One theory is that the especially high protein turnover in the photoreceptors is responsible for their unique susceptibility to heterozygous mutations in splicing factors[44]. The discs in the outer segments have constant turn over as the older discs are phagocytized by the RPE[45]. This requires a constant replenishment of phototransduction proteins and disc structural

proteins. If splicing is impaired, it is hypothesized that not enough of these proteins are produced resulting in destabilized discs or dysfunctional phototransduction. Interestingly, while mutations in proteins involved in most of these cellular functions are either recessive or a mix of dominant and recessive, all known mutations in splicing factors that cause retinitis pigmentosa are dominant due to haploinsufficincy[46].

Genes expressed primarily or exclusively in the RPE can also cause retinal degeneration[47]. The RPE provides trophic support to the photoreceptors[48]. Mutations in genes involved in metabolic support to the retina will have a direct impact on photoreceptor viability. The RPE also plays a critical role in the retinoid cycle, which is essential in order to provide 11-cis-retinal (a necessary substrate for phototransduction) to the photoreceptor outer segments[49]. During phototransduction, 11-cis-retinal is converted to all-trans-retinal and the RPE must convert it back to 11-cis-retinal before sending it back to the phtoreceptors[49]. This process can be disrupted by mutations in enzymes and transport proteins in the RPE including *RPE65*, *IRBP*, *RDH5* and *LRAT*[50]. The RPE also plays a necessary role in disc turnover[51]. Preventing efficient phagocytosis of photoreceptor outer segments (such as by mutations in *MERTK*) can be toxic to the photoreceptors[52].

Mutations in the choroid, the vascular layer of the eye, can also cause retinal degeneration but are much less common than mutations in the RPE or photoreceptors. *CA4* mutations cause RP by preventing proper pH balance in the eye[53]. Gyrate atrophy, which causes choroid and retinal atrophy, is caused by mutations in *OAT*. Ornithine Aminotransferase (OAT) deficiency causes a buildup of ornithine which is toxic to the

choroid and RPE[54]. These causes of retinal degeneration are very rare and it is unlikely that many of the unidentified causes of retinal degeneration are from genes expressed primarily in the choroid.

Next generation sequencing for inherited retinal degeneration

It used to be very expensive and time consuming to identify the disease causing mutation(s) in patients with retinal degeneration. Unlike diseases like cystic fibrosis that only have one gene to screen for mutations, over 140 genes have been identified that cause retinal degeneration with a significant number of genes still unknown[33]. With the advent of next generation sequencing (NGS), sequencing a large number of genes has become much quicker and more affordable than it was previously[55]. Clinically, APEX microarray chips are often used to identify specific mutations in specific genes that are known to cause disease[56]. This can be an effective tool if the patient has one of the mutations screened for on the chip. However, chip arrays miss new mutations in the genes that are being screened since they look specifically for the identified pathogenic single nucleotide polymorphisms (SNPs)[57]. Sequencing is a much more thorough way to screen for mutations in the genes that have already been identified[56]. Furthermore, being able to sequence the entire genome facilitates novel gene discovery and will help to fill in the gaps of our current knowledge about the genetics of retinal degeneration.

Novel gene discovery is beneficial for both translational and basic science. It allows us to better identify patient populations for clinical trials and to prioritize genes based in part on the size of the potential patient population. By discovering the missense mutations

that are tolerated or not tolerated, we can better understand which domains are important to the function of the protein. By finding the genes in which mutations cause retinal degeneration, we gain a better understanding of how the retina works and what function those proteins have in the retina.

Whole genome sequencing (WGS) is the most thorough way to screen for novel mutations[58]. It allows us to find deep intronic mutations, mutations in splice sites, mutations in regulatory regions, as well as mutations in the actual genes[59,60]. However, that level of genomic coverage is not always needed. The majority of disease causing mutations are in the exome, the protein coding portion of DNA. The exome makes up only about 2% of the human genome. Initial screening with whole exome sequencing (WES) could eliminate the need for more extensive sequencing in most patients. WES can detect both mutations in the exome as well as the adjacent splice sites[61]. Both WGS and WES can identify SNPs and small insertions and deletions. One major caveat of next generation sequencing is that it is not ideal for finding large insertions and deletions. WGS is better at detecting copy number variants (CNVs) than WES, but both require a significant depth of coverage to detect CNVs reliably.

WES analysis has two main stages. First, the reads must be mapped onto the human genome and the variants identified and annotated. This part of the process can now be largely automated and the output is a list of areas where the reference genome is different from the patient's genome, annotated with the observed frequency in that variant in databases like ExAC and the 1000 Genomes Project as well as the expected effect on the protein[62-66]. The second stage of WES analysis is currently the bottleneck in the

process[67]. Sorting through the variants to identify likely pathogenic variants cannot yet be automated and requires a lot of assumptions to create a manageable list of candidate genes[68]. Some filters can be applied easily to the dataset without a high likelihood of filtering out the pathogenic variant[69]. For example, filtering by frequency of the variant is a good first step. When dealing with a rare monogenic disease, a variant with a high frequency is unlikely to be involved[69]. Removing all synonymous variants, variants that have no effect on protein sequence, is another filter that is unlikely to remove pathogenic variants[68]. Filtering for genes expressed in the affected tissue can also be beneficial. However this list is still too long to go through every variant by hand to check for likely pathogenic variants. Developing effective filters for retinal degeneration will help to more quickly and accurately identify the pathogenic mutations in the patients screened with WES.

Our goal is not only to identify the cause of retinal degeneration but ultimately to treat it. Gene therapy is a promising approach for treating hereditary retinal degeneration. If there is a loss of function mutation, a new copy of the gene can be inserted into the cell so that the protein is produced and degeneration is halted. The first gene therapy for retinal degeneration recently completed phase III clinical trials[70]. It is for LCA patients with mutations in *RPE65,* which is an ideal candidate for gene therapy. The gene has a short coding sequence, does not require large amounts of expression to be effective, is not toxic when overexpressed, is expressed in an easy to transduce cell type and has a relatively wide window for treatment[71-73]. Additionally, treating mutations in the RPE does not require as high a transduction efficiency as a mutation in photoreceptors. A single RPE

cell supports multiple photoreceptors (with a 20:1 photoreceptor:RPE ratio in the fovea) thereby saving more cells per RPE cell transduced, while correcting a defect in the photoreceptor would mainly help the cells that are actually transduced[74].

Unfortunately, not all genes are good candidates for gene augmentation therapy. Some genes, like *USH2A* and *ABCA4*, are simply too big for most current vectors. Adeno-associated virus (AAV), the most common vector and the one used in the *RPE65* trial, can only package about 4.7 kb which includes the ITRs, promoter and cDNA sequence of the gene[75]. *USH2A* is over 15 kb while *ABCA4* is over 6 kb[76,77]. Even without taking into account the space needed for a promoter it is obvious that these genes cannot be packaged in AAV. Furthermore, some genes are toxic if overexpressed or require a high level of expression to prevent degeneration[78]. Currently, we do not have the ability to finely tune expression levels to make them match the exogenous expression levels, although we can select weaker, cell specific promoters to limit toxicity. Unfortunately, using a weaker promoter would risk lowering the expression level below the therapeutic threshold. Treating a dominant gain-of-function mutation is even more complicated. It requires specific knock down of the mutant allele, which may vary from the wildtype allele by a single nucleotide. The clustered regularly interspaced short palindromic repeats (CRISPR) system or RNA interference (RNAi) may be able to be used in these cases, but depending on the mutation, it may not be possible to achieve the needed level of specificity with current technology[79,80]. Finally, there are too many different genes, some of which affect only a handful of patients, for it to be feasible to develop a specific gene therapy for each gene.

Optogenetic therapy to restore vision

Optogenetic therapy is a generic treatment that has the potential to restore visual function to the retina regardless of the genetic cause of disease[81]. Although it is unlikely to result in the same level of visual restoration as gene augmentation due to the inherent limitations in image resolution, it would be applicable for every genetic cause rather than only one and would not be impacted by the number of patients with a particular mutation, size of the defective gene or the narrow treatment window to preserve photoreceptors[81]. Optogenetic therapy works by inserting a light sensitive ion channel into the desired cell type so that it hyperpolarizes or depolarizes the cell directly in response to light[82,83]. Essentially, the goal of optogenetic therapy is to turn other cell types in the retina into photoreceptors after the photoreceptors have died or stopped functioning.

There are several potential targets for optogenetic therapy. The four most promising possibilities are: remnant cone cell bodies, ON bipolar cells, AII amacrine cells and ganglion cells[81]. Targeting halorhodopsin to remnant cone cell bodies induces them to hyperpolarize in response to light, mimicking their normal light response[84]. Remnant cone cell bodies are cone photoreceptors which no longer have outer segments. They would not be as sensitive to light as normal photoreceptors or have as large of a response, but all retinal processing would be preserved. Early studies in mice have successfully used this approach to restore light mediated behavioral responses as well as the electrophysiological response to light in the retina[85]. However, eventually the remnant cone cell bodies would likely die, thereby limiting the long term viability of this

treatment option. Optogenetic therapy is designed to restore function, not to halt or even slow cell death.

The second most attractive target is ON bipolar cells. If channelrhodopsin is expressed in ON bipolar cells, the cells depolarize in response to light[86-88]. Normally the ON bipolar cells would depolarize in response to the decrease in glutamate release from the photoreceptors. Like all optogenetic therapy, the light sensitivity is lower than what is seen in healthy photoreceptors, but with the advancements being made in engineering more sensitive channelrhodopsins this will become less of a problem[89,90]. We can specifically target ON bipolar cells by using the *GRM6* promoter[86]. This helps to retain more of the retinal processing since the OFF pathway would not be activated, which could confuse or cancel out the signal.

Targeting the AII amacrine cells would allow activation of the ON pathway as well[81]. AII amacrine cells connect the rod bipolar cells to the cone bipolar cells[91]. They depolarize in response to glutamate release from the rod bipolar cells[92]. AII amacrine cells are connected to ON cone bipolar cells by gap junctions, causing the ON cone bipolar cells to depolarize as well[92]. In addition to the excitatory output to the ON pathway, AII amacrine cells form inhibitory synapses with OFF bipolar cells thereby simultaneously exciting the ON pathway and inhibiting the OFF pathway[92]. Targeting AII amacrine cells may allow for more inhibition of the OFF pathway while retaining the ON pathway stimulation.

Ganglion cells are the final target for optogenetic therapy[93,94]. Ganglion cells are the cells responsible for retinal output to the brain. Targeting ganglion cells would remove all or most retinal processing of the visual signal. However, retinal degeneration and retinal remodeling are less likely to affect the quality of the visual signal over time. Currently we do not have promoters that are specific to ON vs OFF ganglion cells and instead must target both and hope that the neuroplasticity is able to adjust to the new input (an OFF cell becoming an ON cell).

The final strategy for optogenetic therapy is to use a ubiquitous promoter that will turn any cell transduced into a photoreceptor cell[85,95]. The advantage of this strategy is the higher expression levels achieved by ubiquitous promoters. More cells total would be responding to light which could increase the sensitivity of the treated retina. However, both the ON and OFF pathways would be activated simultaneously. Activating an excitatory and inhibitory cell at the same time could cause them to cancel out or at least diminish the signal. We do not know the limits of retinal plasticity and brain plasticity so it is undetermined whether a completely non-targeted therapy would result in a usable retinal signal or if the brain would interpret it as essentially gibberish.

Retinal remodeling and optogenetic therapy

One of the most important factors for successful optogenetic therapy is understanding the effect of retinal remodeling on the therapy (and vice versa). There is a lot that we still do not understand about retinal remodeling. It used to be assumed that the inner retina remained largely unchanged during degeneration. This is now known to be untrue. At

late stages of retinal degeneration the inner retina can undergo profound changes[96-101]. Studies in *rd10* mice have been done which detail some of the effects of retinal remodeling[102,103]. *Rd10* mice have a fast rate of retinal degeneration with only a single layer of photoreceptors remaining at P45[102]. The first sign of retinal remodeling is retraction of the rod bipolar cell dendrites and mislocalization of mGluR6 from the dendrites to the cell body and axon[103]. While inner retinal cell death is much slower than photoreceptor degeneration, about a quarter of the rod bipolar cells and horizontal cells degenerated after 9 months in the *rd10* mice[103]. Various cell types start to migrate to new positions, including RPE cells invading the neural retina, ganglion cells migrating into the inner plexiform and inner nuclear layers, and bipolar cells migrating up to the top of the inner nuclear layer[98]. Ectopic synapses form between all cell types and the expression profiles of the cells change[98,104]. These changes have the potential to greatly affect the long term efficacy of optogenetic therapy.

Overall, in order to better treat retinal degeneration, we need to thoroughly understand both the genetics of retinal degeneration as well as the changes that occur to the retina over the course of retinal degeneration. Tools like whole exome sequencing and optogenetics are allowing us to evaluate the degenerate retina more thoroughly than was previously possible. Here, we develop a screening process for whole exome sequencing data to identify pathogenic mutations using genes known to be linked to retinal degeneration and identify subjects who are good candidates for novel gene discovery. We also evaluate the utility of the *GRM6* promoter for optogenetic therapy targeting ON bipolar cells. While a generic treatment is needed for retinal degeneration, our data

shows that the *GRM6* promoter does not remain specific to bipolar cells in advanced stages of retinal degeneration. The work presented here will help us to better understand both the genetic cause and subsequent genetic regulation within the retina and hopefully bring us closer to being able to effectively treat inherited retinal degeneration.

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Chapter 2: Prescreening Whole Exome Sequencing Results from Patients with Retinal Degeneration for Variants in Genes Associated with Retinal Degeneration

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Abstract:

Background: Accurate clinical diagnosis and prognosis of retinal degeneration can be aided by the identification of the disease causing genetic variant. It can confirm the clinical diagnosis as well as inform the clinician of the risk for potential involvement of other organs such as kidney disease. It also aids in genetic counselling for affected individuals who want to have a child. With the advent of next generation sequencing, identifying pathogenic mutations is becoming easier, especially the identification of novel pathogenic variants.

Methods: We used whole exome sequencing on a cohort of 69 patients with various forms of retinal degeneration. All potential pathogenic variants were verified by Sanger sequencing and, when possible, segregation analysis of immediate relatives. Potential variants were identified by using a semi-masked approach in which rare variants in candidate genes were identified without knowledge of the clinical diagnosis (beyond "retinal degeneration") or inheritance pattern. After the initial list of genes was prioritized, genetic diagnosis and inheritance pattern were taken into account.

Results: We identified the likely pathogenic variants in 64% of the subjects. 7% had a single heterozygous mutation identified that would cause recessive disease and 13% had no obviously pathogenic variants and no family members available to perform segregation analysis. Eleven subjects are good candidates for novel gene discovery. Two de novo mutations were identified that resulted in dominant retinal degeneration.

Conclusion: Whole exome sequencing allows for through genetic analysis of candidate genes as well as novel gene discovery. It allows for an unbiased analysis of genetic variants to reduce the chance that the pathogenic mutation will be missed due to incomplete or inaccurate family history or analysis at the early stage of a syndromic form of retinal degeneration.

Keywords: retinal degeneration, genetic diagnosis, retinitis pigmentosa, Leber congenital amaurosis, cone-rod dystrophy, whole exome sequencing

Introduction:

Many forms of retinal degeneration result from genetic mutations including Leber's congenital amaurosis (LCA), retinitis pigmentosa (RP), Stargardt's disease and cone-rod dystrophy. Many different genes have been discovered that, when mutated, lead to retinal degeneration. For LCA alone, mutations in 18 different genes have been discovered to be pathogenic, with about 30% of cases having no known genetic cause[1,2]. Retinitis pigmentosa can be caused by mutations in any one of over 80 different genes with many cases still having an unknown cause[3-5]. While there has been great progress in identified disease-causing mutations for retinal degenerative diseases, this presents a staggering problem to the clinician: what is the most efficient and cost-effective test for identifying the genetic diagnosis?

Discovering genes that cause retinal degeneration when mutated and the specific mutations that do or do not cause disease is important to advancing the field. If the pathogenic mutation(s) can be identified in every patient with retinal degeneration we will know how prevalent those mutations are, will be able to provide accurate prognoses, and will be better able to define potential patient populations for clinical trials. With the growing potential of novel strategies using gene editing (such as use of Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR)) to correct specific genetic mutations, identifying not only the gene but the specific mutations in each patient is becoming even more important. Identifying pathogenic variants in retinal degeneration benefits basic science as well as clinical research. By identifying the genes that result in retinal degeneration when mutated, we gain a better understanding of how the retina

works, which proteins are necessary for the various cellular processes that take place in the retina, and how they interact. By defining the specific mutations that are pathogenic (and which ones are tolerated) we better understand the function of those proteins and domains. This information can also be used to develop gene-based treatments (i.e. gene therapy).

The present report reviews our own experience in seeking genetic diagnoses for retinal degeneration patients seen over a 5 year period at ophthalmology clinics at the University of Pennsylvania School of Medicine and The Children's Hospital of Philadelphia as well as other patients who self-referred who agreed to participate in our molecular genetic research study. We carried out preliminary screens of these individuals in an attempt to identify known disease-causing variants. We followed up with whole exome sequencing (WES). Finally, we assessed the efficiency of making the correct genetic diagnosis using these techniques.

Methods:

Recruitment: Individuals seen in the Scheie Eye Institute and The Children's Hospital of Philadelphia retina clinics who were found to have a degenerative condition (and first degree relatives) were invited to submit a blood sample for molecular genetics research testing. Study procedures were approved by the University of Pennsylvania IRB (#808828) and each individual provided signed informed consent.

Whole Exome Sequencing:

Purified, target enriched genomic samples from 69 patients with various form of retinal degeneration (including LCA, RP, Stargardt's disease, cone rod dystrophy and achromatopsia) to the Penn Genome Frontiers Institute (PGFI) were evaluated by whole exome sequencing (see table 2-1). Samples had previously been screened using Asper Ophthalmics (Tartu, Estonia) arrayed primer extension multi-gene panels for the relevant disease and no disease-causing variants had been identified. Target enrichment was performed with the Agilent SureSelect target enrichment system (Agilent Technologies, Santa Clara, CA)and the sequencing was performed on Illumina HiSeq2000 (San Diego, CA).

Mapping and variant identification:

Mapping and variant identification was performed using Galaxy[6]. The FASTQ files for each patient were mapped to the reference human genome (hg18) using Burrows-Wheeler Aligner for Ilumina (BWA for Ilumina). Variants were identified using freebayes[7]. Variants were annotated using Annovar[8].

Identifying variants of interest:

A semi-masked analysis was used to identify potentially pathogenic mutations without knowledge of the type of retinal degeneration. We created a curated list of genes linked to any form of retinal degeneration and used a python script (www.python.org) to create a customized list of potential pathogenic variants for each patient. That list consisted of retinal degeneration genes with variants with an allele frequency of less than 0.05. Those

mutations were then prioritized based on mutation type and known facts about the mutations from dbSNP (https://www.ncbi.nlm.nih.gov/projects/SNP/) and the Universal Protein Resource (UniProt; http://www.uniprot.org) (known to be benign, known to be pathogenic, novel variant, etc.). We then checked if the variants were consistent with the clinical diagnosis and performed segregation analysis when possible.

We verified the possible pathogenic mutations in the patients and relatives via PCR using Phusion polymerase (New England Biolabs; Ipswich, MA) and custom primers made by Invitrogen (Carlsbad, CA) followed by Sanger sequencing by the Penn Genomics Analysis Core. The sequences were visualized using SnapGene software (from GSL Biotech, Chicago, IL; available at snapgene.com).

Results: We identified the pathogenic mutations in 44 of the subjects (65%). 66 different mutations were found in 26 different genes. 18 of these mutations are completely novel and not listed in dbSNP. 11 more are extremely rare, have no prediction on pathogenicity and are not in any published study (See supplement for specific mutations in each form of retinal degeneration).

De Novo Mutations:

De novo mutations pose a particular problem when trying to identify pathogenic mutations. Dominant *de novo* mutations appear to be recessive when looking at the family history. We found two *de novo* mutations in our patient cohort. The first was a novel frameshift mutation in *OTX2*, a known mutational hotspot, in a patient with Leber's congenital Amaurosis (LCA; Table 2-2; JB275). *OTX2* is a transcription factor that is

essential for development of the brain and retina. All known mutations are autosomal dominant. Knockout of *OTX2* is embryonic lethal in mice due the absence of the forebrain and midbrain[9]. *OTX2* is essential for photoreceptor differentiation as well as pituitary development. Knockout of *OTX2* in mouse retinal cells results in a failure to develop photoreceptors[10]. *OTX2* mutations can result in syndromic microphthalmia, combined pituitary hormone deficiency, and early-onset retinal dystrophy with or without pituitary dysfunction[11-13]. The frameshift mutation was not present in either parent.

Another subject presented with cone dystrophy beginning at age 15. Neither parent had any form of retinal degeneration so it was presumed that the genetic cause was recessive. Upon analysis of the WES dataset, we found that the proband was heterozygous for a p.Y99C mutation in GUCA1A (Table 2-2, JB185), a well characterized dominant mutation leading to cone dystrophy[14]. Neither parent carried the p.Y99C mutation. Paternity was confirmed using the whole exome sequencing results of the parents and proband, indicating that the p.Y99C mutation was a *de novo* mutation.

Identification of KIF7 as a candidate gene for cone dystrophy:

A 5yo boy with cone dystrophy was found to have a compound heterozygous mutation in *KIF7* (p.H1115Q and p.Q834R)(Table 2-2; JB307). *KIF7* is a cilia gene that plays an important role in hedgehog signaling and microtubule stability[16,17]. KIF7 localizes to the primary cilia, specifically the distal tip[18]. Mutations in *KIF7* cause the cilia to become longer than normal and disorganized[18]. Knockout of KIF7 in mice is neonatal lethal[17]. Mutations in *KIF7* have been shown to cause severe ciliopathies including Joubert

syndrome, hydrolethalus syndrome, acrocallosal syndrome, Meckel-Gruber syndrome and BBS[19]. Both variants in the patient in this study have been previously identified as hypomorphic variants and have been seen in patients with BBS, Meckel-Gruber syndrome and hydrolethalus syndrome[20]. Typically, when a missense mutation in *KIF7* leads to disease, the other allele is a null allele with either a truncation or frameshift mutation[20,21]. In this case, both alleles are rare hypomorphic variants (allele frequency in 1000 Genomes are 0.0010 and 0.0058 for p.Q834R and p.H1115Q respectively). In addition to the compound heterozygous mutations, the subject was heterozygous for mutations in several other cilia genes, including a pathogenic mutation in *BBS1*. Ciliopathies have been shown to have a range of severities depending on the mutation in the gene. For example, *CEP290* mutations can result in retinal degeneration alone (specifically LCA), syndromic ciliopathies (Joubert syndrome or Meckel syndrome) and neonatal lethal ciliopathies. This range appears to be due the amount of functional CEP290 that remains[22]. *KIF7* could be similar to *CEP290* in that severe mutations like truncations and frameshifts result in more severe ciliopathies while compound heterozygosity of hypomorphic variants may result in only retinal degeneration.

Additional mutations:

Some of the patients had potentially pathogenic mutations in additional genes linked to retinal degeneration. Most of these additional mutations were heterozygous and therefore unlikely to be the disease causing mutation in the patient. However, it is possible that the heterozygous mutations, which can be tolerated in isolation, add to the mutational load and modify the patient's phenotype[23]. Five subjects had an unusually high number of

additional mutations (see tables 2-4 and 2-5). Some of the mutations are in genes that interact or are in the same pathway, increasing the chances of epistatic effect.

No disease causing variants identified:

We were unable to conclusively determine the disease causing variants in 20 subjects in the study. Five of these had a presumably recessive disease with one pathogenic or likely pathogenic variant identified that is consistent with their disease phenotype (see table 2-3). These patients should be screened for copy number variants (CNVs) in the gene identified or possibly for intronic variants. Of the remaining 20 subjects, 9 do not have family members willing to enroll in the study. Since we did not find any obviously pathogenic variants in the initial screening and are unable to perform segregation analysis for the variants identified, we are unable to determine the pathogenicity of the variants identified. One of these subjects has many known pathogenic, likely pathogenic and novel variants but all are inconsistent with the diagnosis of adRP and we are unable to perform segregation analysis to narrow down the disease causing variant(s) (see table 2-5). The remaining 10 subjects should be analyzed for novel genes that could cause retinal degeneration (see figure 2-1).

Discussion:

Whole exome sequencing is a powerful tool to help identify new mutations in patients who test negative for the known pathogenic mutations for retinal degeneration. However, the majority of mutations in these patients are in genes already linked to retinal degeneration. Forty-four of the sixty-nineprobands in our study, all whom had had

negative screenings using gene panels for their disease, were found to harbor pathogenic mutations in genes already linked to retinal degeneration. Thus, we found that if we failed to find the pathogenic mutation in a patient with retinal degeneration using standard APEX microarrays for their disease, it could be more time and cost efficient to use targeted sequencing of all genes linked to retinal degeneration rather than whole exome sequencing. Using a targeted sequencing approach would also allow good coverage of the area of interest without the expense of deeper coverage of the whole exome. When we restricted our analysis of the whole exome sequencing data to genes linked to retinal degeneration, it allowed us to find novel mutations in those genes as well as mutations that cause a different form of retinal degeneration.

WES is a powerful tool for identifying pathogenic mutations in genes by quickly identifying all variants in a patient's exome. It sequences the entire protein coding portion of the genome, which is about 2% of the total genome. Since the non-coding portion is not sequenced, it is cheaper than whole genome sequencing (WGS) and allows for greater depth of coverage. The large majority of disease-'causing variants are in the exome, so although intronic mutations are missed, it is still usually more cost effective to use WES and follow up with WGS or targeted sequencing if WES fails to identify the cause of disease.

Although the size of the dataset generated by WES is smaller than WGS, sorting through the large amount of data generated to find the relevant variants is still a challenge. Filtering the dataset based on inheritance pattern, disease phenotype, and other predetermined criteria can make the dataset more manageable but also risks filtering out

pathogenic variants. De novo mutations can result in a dominant mutation in a patient whose disease is presumed to be recessive. Dominant mutations can also be revealed by findings of non-paternity (and concomitant incorrect family history). Diagnosis is based on qualitative assessment and psychophysical testing and many forms of retinal degeneration have significant phenotypic overlap with each other. A patient can be diagnosed with a non-syndromic form of retinal degeneration when they have a syndromic form if they have not yet developed extra-ocular symptoms. In these cases it is easy to filter out pathogenic variants if the filters are too strict.

Limiting our initial screening to the genes that are known to cause one particular form of retinal degeneration would have been unnecessarily restrictive. It would have placed too much confidence in the accuracy of the clinical diagnosis when there is significant overlap between different forms of inherited retinal degeneration. Further, at both early and late stages of retinal degeneration, it may be difficult to distinguish one form of disease from another. The possibility of de novo mutations and inaccurate family history makes it impossible to determine the inheritance pattern with complete certainty even in the best case scenario. There are also multiple genes that are linked to multiple forms of retinal degeneration, with the mechanism by which they can differentially affect rods and cones still not understood. It is entirely possible that a gene in which mutations are known to cause only RP or LCA currently could in fact also have mutations that lead to cone dystrophy. When one adds in the possibility of digenic cause and epistatic effects, it becomes even clearer that limiting analysis by the form of retinal degeneration is overly simplistic and can lead to missed pathogenic mutations.

So far, most of the mutations we have found that lead to retinal degeneration follow typical Mendelian genetics. However, many patients with retinal degeneration do not have a known cause. It is probable that at least some of these cases will be found to be due to the additive or epistatic effects of mutations in more than one gene. Especially in the case of a presumed recessive inheritance pattern, the possibility that the cause is polygenic rather than monogenic cannot be discounted. In light of that possibility, screening all genes with mutations known to cause or contribute to retinal degeneration is important. The fact that these genes code for proteins vital to the proper functioning of the retina has already been established. A mutation or variant in a gene that reduces the ability to function (but not so much that it is sufficient to cause disease in the general population) might very well add to the mutational load that predisposes someone to retinal degeneration.

By using a semi-masked analysis in our screening, we were able to easily identify two patients with dominant de novo mutations as well as several with an inaccurate or incomplete incoming diagnosis. Many of the subjects in our study self-reported the initial diagnosis, which decreases the initial accuracy. However, there is some inherent unreliability in the initial diagnosis for early stage retinal degeneration, particularly if this is made by a non-specialist. Some forms of retinal degeneration, like retinitis pigmentosa, could be a part of a syndromic form of retinal degeneration like BBS. By not taking into account the type of retinal degeneration until after the possible mutations have been identified, we eliminate the possibility of filtering out obviously pathogenic variants before we even begin the analysis.

One cautionary finding from our study is that there were several patients that had areas of low coverage in genes of interest. One patient with Stargardt disease had a gap in coverage in *ABCA4 that* masked the pathogenic mutation. Such gaps need to be filled in with Sanger sequencing. A gap in coverage can complicate analysis if not detected as it would essentially show no mutation in the area with no or low coverage.

We believe that all subjects should undergo appropriate psychophysical testing in order to refine the clinical diagnosis and then undergo a panel mutation screening for that diagnosis before being enrolled in a study for novel gene detection. This should be followed by targeted exome sequencing of all genes linked to retinal degeneration. In our study, 64% of the subjects were eliminated from further analysis after screening only genes linked to retinal degeneration. Subjects with a recessive disease in which only 1 pathogenic mutation is found (7% of the patients in this study, 20% of those without a genetic diagnosis) should be screened for CNVs and intronic variants in that gene. After eliminating those with no relatives enrolled in the study, this would leave only 16% for a more in depth analysis using whole exome sequencing.

The subjects with an unusually high mutational load merit further study as well. We tend to view sporadic cases of early onset retinal degeneration as being due to an autosomal recessive mode of inheritance, or occasionally a *de novo* mutation. Modifying alleles are sometimes identified which increase the severity of the disease phenotype, like the p. A229T variant in *RPGRIP1L*[24]. However, the consequence of multiple heterozygous variants that can be pathogenic if there is a second mutation in the same gene has not been studied. Screening the unaffected relatives of the three subjects with an unusually

high mutational load could help illuminate how many mutations can be tolerated. Studying the progression of retinal degeneration in these subjects can help show whether the additional mutations result in a more severe phenotype or have no obvious effect.

Conclusion:

Whole exome sequencing is a very effective approach for identifying novel mutations and narrowing down candidate genes for further analysis especially when paired with targeted analysis based on known retinal pathways. If WES does not yield a molecular genetic diagnosis or a likely candidate for further analysis, whole genome sequencing can be used to identify non-coding (intronic) mutations. While analysis of the parents and immediate family of the proband is not always required for identification of pathogenic variants, it does increase the likelihood of identifying pathogenic variants and could be needed if the mutations are not obviously pathogenic.

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Supplement:

Syndromic:

Three siblings in one family were diagnosed with RP. WES revealed two variants in *BBS4* in the proband (Table 2-2; JB319). The first mutation was a frameshift mutation likely resulting in nonsense mediated decay. The second mutation was p.R295G. While the arginine to glycine mutation is novel, an arginine to proline mutation at the same location has been previously reported to be pathogenic, making it very likely that the glycine substitution would be pathogenic as well[25]. Mutations in *BBS4* have been shown to cause Bardet-Biedl syndrome (BBS) but not non-syndromic retinitis pigmentosa. Segregation analysis confirmed that all three siblings were compound heterozygous for the *BBS4* mutations and clinical analysis showed that their symptoms (retinitis pigmentosa, obesity and learning difficulties) were consistent with mild BBS. Renal dysfunction, polydactyly, and male hypogonadism are also commonly seen in Bardet-Biedl syndrome but were absent in this family[26].

Another subject had Usher syndrome, a condition characterized by hearing loss and retinitis pigmentosa. Clinical testing failed to reveal any known mutations leading to Usher syndrome. WES revealed two novel frameshift mutations in *GPR98* (Table 2-2; JB324), a gene known to cause Usher syndrome when mutated[27]. Another subject with

Usher syndrome was found to have two mutations in *USH2A* (Table 2-2, JB252) that had been already classified as pathogenic.

Stargardt, cone dystrophy and achromatopsia:

Stargardt disease is a childhood onset macular degeneration and is most commonly caused by mutations in *ABCA4.* Characteristic yellow flecks are typically seen under the macula during a fundus exam. Five subjects presented with Stargardt disease and were found to have mutations in *ABCA4* (Table 2-2: JB260, JB333, JB358, JB16, JB320). Six of the mutations had been previously characterized and determined to be pathogenic while three novel frameshift mutations were found.

Autosomal dominant Stargardt disease is caused by mutations in *PROM1*. Interestingly, the dominant mutations seem to cause primarily cone degeneration while the recessive mutations can cause either rod or cone degeneration. The dominant mutations in *PROM1* are typically gain of function missense mutations while the recessive mutations are loss of function mutations including frameshift mutations, splice site mutations and truncations. Two loss of function variants were identified in patients with either Stargardt disease or cone dystrophy (see table 2-3; JB189, JB241). A second variant was not identified in either case. Since a dominant inheritance pattern was not established in these families, the pathogenicity of these variants is currently undetermined.

One subject diagnosed with Stargardt disease was found to have a heterozygous p.V242M variant in *CRX* (Table 2-2; JB195). This mutation is reported to be pathogenic and cause autosomal dominant cone rod dystrophy (CORD)[28]. CRX is a transcription

factor that is required for the development and maintenance of cone photoreceptors[29]. While many *CRX* mutations have been shown to reduce the ability of CRX to activate the rhodopsin promoter in vitro, the p.V242M mutation did not appear to affect the ability of CRX to activate the rhodopsin promoter, casting some doubt on the pathogenicity[30]. It is possible that the p.V242M variant is pathogenic but has a different functional effect. The variant did not segregate with disease in the family in this study. Both the mother and half-sister are heterozygous for the variant but do not have retinal degeneration. It is possible that the p.V242M variant contributes to mutational load or digenic cause for disease, but based on the lack of segregation and lack of demonstrated functional effect, the variant is probably benign.

Another subject presented with severe retinal degeneration that was classified as either achromatopsia or LCA (Table 2-2; JB301). The proband had two novel mutations in *GNAT2*, a frameshift mutation and a missense mutation. *GNAT2* encodes the alpha subunit of cone transducing which is essential for phototransduction in cones[31]. Truncations are the most common pathogenic mutations in *GNAT2*, but pathogenic missense mutations in *GNAT2* have been seen in both patients and mice[32,33]. Complicating the interpretation of the sequencing results for this subject, she had a p.A249E heterozygous mutation in *GDF6* that has been reported to be autosomal dominant with incomplete penetrance[34-36]. This variant has been reported to lead to LCA, microphthalmia and Klippel-Feil syndrome. Notably, this subject does not have any skeletal defects that are sometimes reported to be associated with the p.A249E variant. The mother, who does not exhibit any symptoms of retinal degeneration is positive for

the missense variant in GDF6. Therefore, we determined that the *GNAT2* mutations are the most likely genetic cause in this subject, with the *GDF6* mutation potentially acting as a modifying allele and increasing the severity or playing no role in retinal degeneration in this subject.

Another subject had an incoming diagnosis of LCA that was changed to cone dystrophy upon examination. The diagnosis was further modified to achromatopsia after whole exome sequencing revealed two heterozygous mutations in *CNGB3*, one of which was novel (Table 2-2; JB426).

LCA:

LCA is a severe retinal degeneration which is symptomatic before the first year of age[37]. The retinal degeneration eventually includes cone loss as well. It is one of the most severe forms of hereditary blindness. In this study we found mutations in five different genes that resulted in LCA (see table 2-2). Nine of the mutations (in *ABCA4, CNGB3, KCNJ13*, and *PROM1*) were novel.

CEP290 mutations are the most common cause of LCA. CEP290 is essential for cilia development and maintenance[38,39]. Mutations in *CEP290* cause many different ciliopathies including Joubert syndrome, Bardet-Biedl syndrome, Meckel syndrome and Senior-Loken syndrome[22,39]. We found a novel splice site mutation in *CEP290* in one patient with LCA (Table 2-2; JB165). The second mutation was the deep intronic splice mutation[40]. This case illustrates one of the limitations of WES for finding pathogenic mutations. Whole exome sequencing did not reveal the deep intronic mutation. Since the

CEP290 c.2991+1655A>G mutation is the most common mutation leading to LCA, the patient had been screened for it previously. If the mutation had not already been identified and specifically looked for, we would not have identified the second mutation or been able to determine if the *CEP290* splice site mutation was actually disease causing in this case or if the patient was simply a carrier for the mutation.

Mutations in *PRPH2* tend to be autosomal dominant due to haploinsufficiency[41]. Typically these mutations cause either retinitis pigmentosa or macular degeneration[42,43]. Homozygous or compound heterozygous mutations are much rarer and cause LCA or early onset RP when they do occur[44]. The parents do not always have any functional vision loss but will usually show signs of mild retinal degeneration when examined. One subject in this study was homozygous for a novel truncating mutation in *PRPH2* (Table 2-2; JB310).

One subject was initially diagnosed with LCA. WES revealed two mutations in *PDE6A* (Table 2-2; JB28) which has been shown to result in RP. Upon re-evaluation the diagnosis was changed to early onset retinitis pigmentosa, which has a later age of onset and typically less severe progression.

Retinitis Pigmentosa:

Retinitis pigmentosa is a progressive form of retinal degeneration that primarily affects rod photoreceptors. It has a later onset than LCA with a typical age of diagnosis of 35 years old. There is a wide range for the age of onset with some patients diagnosed during childhood and other being diagnosed in their 60s or even later[45,46]. RP can be x-linked,

autosomal dominant or autosomal recessive. Over 80 genes have been identified that can cause retinitis pigmentosa and the genetic cause is unknown in almost half of all patients diagnosed[47]. In this study, 10 different genes were found to cause retinitis pigmentosa with two novel mutations (see table 2-2). All of the genes have been previously shown to cause retinitis pigmentosa.

Autosomal dominant RP (adRP) accounts for 15-40% of all cases of RP[3,48]. So far, more than 20 genes have been identified that cause autosomal dominant RP[3,48]. These include *CA4*, *PRPH2* and *PRPF31*. Dominant mutations tend to be pathogenic either due to heterozygous insufficiency or because they cause a toxic gain of function. Mutations in all three of these genes seem to cause disease at least in part due to heterozygous insufficiency as a null allele is sufficient to cause disease. The novel mutation we found in *PRPH2* is an early stop codon so we did not follow up with functional testing. The mutations in *CA4* and *PRPF31* are missense mutations that are near known pathogenic missense mutations in the genes. CA4 mutations have been shown to impair the ability of the cells to regulate intracellular pH[49]. The p.Q254P mutation in *CA4* (Table 2-2; JB42) is very rare and currently classified as a variant with uncertain significance on dbSNP (rs150432787). We were unable to perform a segregation analysis due to the lack of DNA from family members. Interestingly, this patient was heterozygous for several mutations that are known to cause recessive forms of retinal degeneration including *USH2A*, *ABCA4* and *CDH23* as well as novel mutations in several other genes linked to retinal degeneration including *BBS12*, *ROM1* and *GPR98*. This is an unusually high mutational load (see table 2-4).

Figures:

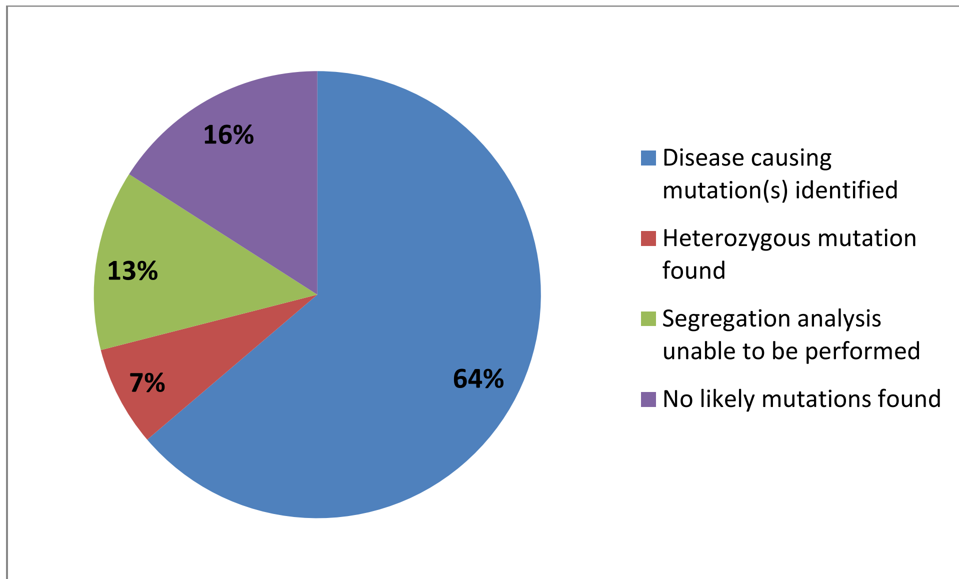

Figure 2-1: Final outcome of initial screening for variants in genes associated with retinal degeneration. Of the initial cohort of 69 subjects with retinal degeneration, the pathogenic variants were identified in 44 subjects, heterozygous mutations consistent with the diagnosis were identified in 5 subjects, 9 subjects had no obviously pathogenic variants and lacked relatives willing or able to enroll in the study to perform segregation analysis on potentially pathogenic variants. Eleven are good candidates for further analysis to find novel genes associated with retinal degeneration.

Incoming Clinical Diagnosis	**Number of patients**
adRP	5
Cone dystrophy	3
CORD	2
LCA	24
Macular dystrophy	1
Nanophthalmos	1
RP	15
Stargardt	11
Usher's syndrome	2
Achromatopsia	1
Vitreoschisis	1
Retinal degeneration (unspecified)	2

Table 2-1: Breakdown of diagnoses included in this study. adRP, autosomal dominant retinitis pigmentosa; LCA, Leber's congenital amaurosis; CORD, cone rod dystrophy; RP, Retinitis Pigmentosa

Subject	Dx	gene	Nucleotide change	Protein change	dbsnp	citation
JB260	Stargardt	*ABCA4*	c.6119G>A	p.Arg2040Gln	rs148460146	[50]
			c.2879del	p.Ala960Aspfs*17	N/A	
JB333	Stargardt	*ABCA4*	c.4363T>C	p.Cys1455Arg	rs758835368	[51]
			c.4666del	p.Arg1556Glyfs*25	N/A	
JB358	Stargardt	*ABCA4*	c.2588G>C	p.Gly863Ala	rs76157638	[52]
			c.3984_3987del	p.His1328Glnfs*60	N/A	
JB16	Stargardt	*ABCA4*	c.5917del	p.Val1973*	rs61751389	[53]
			c.5917del	p.Val1973*	rs61751389	[53]
JB320	Stargardt	*ABCA4*	c.1749G>C	p.Lys583Asn	rs145265791	[51]
			c.4594G>A	p.Asp1532Asn	rs62642574	[54]
JB249	LCA	*AIPL1*	c.834G>A	p.Trp278*	rs62637014	[55]
			c.404_405insA	p.Glu135fs*23	N/A	
JB319	RP (BBS)	*BBS4*	c.513_514insA	p.Ile172Asnfs*18	rs779047261	

			c.883C>G	p.Arg295Gly	N/A	
JB42	adRP	*CA4*	c.761A>C	p.Gln254Pro	rs150432787	
JB165	LCA	*CEP290*	c.2991+1655A>G	splice mutation	rs281865192	[40]
			c.3461+2TA>GT	splice mutation	N/A	
JB290	LCA	*CEP290*	c.2390del	p.Lys797Serfs*2	rs781670422	[56]
			c.2390del	p.Lys797Serfs*2	rs781670422	[56]
JB9	retinal degeneration (Batten Disease)	*CLN3*	c.597C>A	p.Tyr199*	rs267606737	[57]
			c.597C>A	p.Tyr199*	rs267606737	[57]
JB255	LCA (Batten Disease)	*CLN3*	c.883G>A	p.Glu295Lys	rs121434286	[58]
			CNV suspected~			
JB426	CORD (Achromatopsia)	*CNGB3*	c.1148del	p.Thr383Ilefs*13	rs397515360	[59]
			c.1306A>C	p.Ser436Arg	rs748354081	
JB274	LCA	*CRB1*	c.2300T>C	p.Leu767Pro	N/A	
			c.2300T>C	p.Leu767Pro	N/A	
JB375	RP	*CRB1*	c.1576C>T	p.Arg526*	rs114342808	[60]

			c.1429G>A	p.Gly477Arg	rs866822473	[61]
JB402	LCA	*CRB1*	c.2843G>A	p.Cys948Tyr	rs62645748	[62]
			c.3988G>T	p.Glu1330*	N/A	
JB38	RP	*FAM161A*	c.1355_1356del	p.Thr452Serfs*3	rs397704718	[63]
			c.1355_1356del	p.Thr452Serfs	rs397704718	[63]
JB301	LCA vs achromatopsia	*GNAT2*	c.896C>A	p.Ala299Glu	N/A	
			c.720+2T>C	splice mutation	N/A	
JB324	Usher's	*GPR98*	c.14767del	p.Thr4923Profs*8	rs747459491	
			c.17668_17669del	p.Met5890Valfs*10	rs757696771	
JB185	cone dystrophy (ad)	*GUCA1A*	c.296A>G	p.Tyr99Cys	rs104893967	[14]
JB282	LCA	*GUCY2D*	c.2080C>T	p.Gln694*	rs61750164	[64]
			c.1009_1010ins CAGCAGCT	p.Asp337Alafs*61 (p.Pro335_Ser336insSer in cis)		
JB307	Cone dystrophy	*KIF7*	c.A2501A>G	p.Gln834Arg	rs138354681	[20]

			c.3345C>G	p.His1115Gln	rs142032413	[20]
JB32	RP	*NR2E3*	c.119-2A>C	splice mutation	rs2723341	[65]
			c.932G>A	p.Arg311Gln	rs28937873	[66]
JB181	Nanophthalmos; maculopathy	*NR2E3*	c.767C>A	p.Ala256Glu	rs377257254	[67]
			c.119-2A>C	splice mutation	rs2723341	[65]
JB48	RP (ad)	*NR2E3*	c.166G>A	p.Gly56Arg	rs121912631	[68]
JB275	LCA (ad retinal degeneration)	*OTX2*	c.527del	p.Pro177*	N/A	
JB28	LCA (eoRP)	*PDE6A*	c.1705C>A	p.Gln569Lys	rs139444207	[69]
			c.1620+2T>A	splice mutation		
JB33	adRP	*PRPF31*	c.590T>C	p.Leu197Pro	N/A	
JB310	LCA	PRPH2	c.522G>A	p.Trp174*	N/A	
			c.522G>A	p.Trp174*	N/A	
JB167	ad retinal degeneration	PRPH2	c.136C>T	p.Arg46*	rs61755771	
JB46	CORD	PRPH2	c.514C>T	p.Arg172Trp	rs61755792	[43]

JB284	LCA	PROM1	c.2050C>T	p.R684*	rs530749007	
			c.2050C>T	p.R684*	rs530749007	
JB44	adRP	RP1	c.2029C>T	p.Arg677*	rs104894082	[70]
JB372	RP	RP1L1	c.1138G>A	p.Gly380Arg	rs184332984	[71]
			c.1138G>A	p.Gly380Arg	rs184332984	[71]
JB47	RP	RP1L1	c.1270A>T	p.Lys424*	rs770463388	
			c.1270A>T	p.Lys424*	rs770463388	
JB283	LCA	RPE65	c.1067_1068insA	p.Asn356Lysfs*9	rs766074572	
			c.1067_1068insA	p.Asn356Lysfs*9	rs766074572	
JB357	LCA	RPE65	c.1249G>C	p.Glu417Gln	rs62636299	[72]
			c.1102T>C	p.Tyr368His	rs62653011	[73]
JB285	LCA	RPGRIP1	c.767C>G	p.Ser256*	N/A	
			c.1084_1087del	p.Arg363Leufs*11	N/A	
JB124	LCA	RPGRIP1	c.1180C>T	p.Gln394*	N/A	
			c.1180C>T	p.Gln394*	N/A	

JB43	RP	RPGRIP1L	c.171G>T	p.Leu57Phe	rs146925098	
			c.628A>G	p.Asn210Asp	rs146584570	
JB41	RP	USH2A	c.1036A>C	p.Asn346His	rs369522997	[74]
			c.13335_13337del	p.Glu4445_Asn4446delinsAsp	rs775556188	
JB49	RP	USH2A	c.13297G>T	p.Val4433Leu (benign?)	rs111033381	[75]
			c.6713A>C	p.Glu2238Ala	rs41277212	[76,77]
JB252	Usher's	USH2A	c.13207_13208del	p.Gly4403Profs*15	rs746447649	[78]
			c.2299del	p.Glu767Serfs*21	rs80338903	[74,79]

Table 2-2: Pathogenic and probable pathogenic variants in known disease causing genes identified in subjects in this study. Only variants that completely explain the disease phenotype were included in this table. LCA, Leber's congenital Amaurosis; RP, retinitis pigmentosa; BBS, Bardet Biedl Syndrome; CORD, cone-rod dystrophy; eoRP (early onset RP)

subject	DX	gene	Nucleotide change	Protein change	dbsnp	citation
JB200	Stargardt	ABCA4	c.2828G>A	p.Arg943Gln (probable polymorphism)	rs1801581	[54]
JB188	Stargardt	CNGB3	c.2139_2160del	p.Lys714_Gln720del (p.Lys804*in cis)	(rs151039691 in cis)	[59]
JB23	LCA	KCNJ13	c.458C>T	p.Thr153Ile	rs863224884	
JB189	Stargardt	PROM1	c.303+1G>A	splice mutation	rs777673930	
JB241	cone dystrophy	PROM1	c.1623_1624del	p.Y541fs	N/A	

Table 2-3: Heterozygous variants identified in subjects with recessive disease. The variant is most likely pathogenic and is consistent with the diagnosis but we were unable to identify the second mutation. These subjects should be screened for copy number variants and intronic variants. LCA, Leber's congenital Amaurosis

subject	DX	Disease causing gene	Other genes	Nucleotide change	Protein change	dbsnp	citation
JB333	Stargardt	ABCA4	IQCB1	c.772delA	p.Arg258Aspfs*4	N/A	
JB320	Stargardt	ABCA4	RD3	c.16T>C	p.Trp6Arg	rs35649846	[80]
				c.69G>C	p.Glu23Asp	rs34422496	[80]
JB9	Retinal degeneration (Batten Disease)	CLN3	MKKS	c.1015A>G	p.Ile339Val (hom)	rs137853909	[81]
JB375	RP	CRB1	AHI1	c.653A>G	p.Tyr218Cys	rs183936286	[82,83]
				c.3257A>G	p.Glu1086Gly	rs148000791	[82,84]
JB38	RP	FAM161A	RPGRIP1L	c.685G>A	p.Ala229Thr	rs61747071	[24]
JB301	LCA vs achromatopsia	GNAT2	GDF6	c.746C>A	p.Ala249Glu	rs121909352	[36]
JB32	RP	NR2E3	USH2A	c.2137G>C	p.Gly713Arg	rs696723	[85]
				c.10246T>G	p.Cys3416Gly	rs527236140	[86]
JB181	Nanophthalmos; maculopathy	NR2E3	ABCA4	c.2828G>A	p.Arg943Gln	rs61749446	[54,87]
JB184	Nanophthalmos;	NR2E3	ABCA4	c.2828G>A	p.Arg943Gln	rs61749446	[54,87]

	maculopathy						
JB48	RP (ad)	NR2E3	USH2A	c.10246T>G	p.Cys3416Gly	rs527236140	[86]
				c.14419G>A	p.Ala4807Thr	rs534656527	
JB275	LCA (ad retinal degeneration)	OTX2	RP1	c.5673G>T	p.Leu1891Phe	rs139088785	
JB28	LCA (eoRP)	PDE6A	IQCB1	c.962T>A	p.Val321Glu	N/A	
JB284	LCA	RD3	USH2A	c.10451G>A	p.Arg3484Gln	rs771999994	[76]
				c.13709G>A	p.Arg4570His	rs730254	[76]
JB41	RP	USH2A	RP2	c.844C>T	p.Arg282Trp (hom)	rs1805147	[88,89]
JB49	RP	USH2A	RP1	c.4875A>G	p.Ile1625Met	rs757644601	
JB195	Stargardt	unknown	CRX	c.724G>A	p.Val242Met	rs61748459	[28]
JB42	adRP	CA4	ABCA4	c.1140T>A	p.Asn380Lys	rs61748549	[90]
			BBS12	c.617T>G	p.Val206Gly	N/A	
			CDH23	c.1096G>A	p.Ala366Thr	rs143282422	[91]

			GPR98	c.6017G>A	p.Gly2006Asp	rs768201036	
			USH2A	c.2276G>T	p.Cys759Phe	rs80338902	[85]
JB307	Cone dystrophy	KIF7	BBS1	c.1169T>G	p.Met390Arg	rs113624356	[92]
			CEP164	c.4228C>T	p.Gln1410*	rs147398904	
			RPGRIP1	c.1639G>T	p.Ala547Ser	rs10151259	[93]
			CLN3	c.1189G>A	p.Ala397Thr	rs754468227	
			RP1l1	c.6359A>G	p.Glu2120Gly	N/A	
JB284	LCA (RP)	PROM1	USH2A	c.13709G>A	p.Arg4570His	rs730254	[76]
			USH2A	c.13297G>T	p.Val4433Leu	rs111033381	[75]
			USH2A	c.10451G>A	p.Arg3484Gln	rs771999994	
			RP1	c.5840T>G	p.Leu1947Trp	N/A	
			RPGRIP1	c.3341A>G	p.Asp1114Gly	rs17103671	[94]

			BBS1	c.700G>A	p.Glu234Lys	rs35520756	[95]
			ABCA4	c.3602G>T	p.Leu1201Arg	rs61750126	[54]
			RD3	c.69G>C	p.Glu23Asp	rs34422496	[80]
			RD3	c.16T>C	p.Trp6Arg	rs35649846	[80]
			WFS1	c.862G>A	p.Val288Met	rs71537685	
			WFS1	c.1949A>G	p.Tyr650Cys	N/A	
			WFS1	c.2008G>A	p.Gly670Ser	N/A	
JB274	LCA	CRB1	KIF7	c.A2501A>G	p.Gln834Arg	rs138354681	[20]
			BBS1	c.1396G>A	p.Ala466Thr	N/A	
			FAM161A	c.1133T>G	p.Leu378Arg	rs187695569	[96]
			MAK	c.112A>C	p.Lys38Gln	N/A	
			RGS9	c.1351C>A	p.Gln451Lys	N/A	

Table 2-4: Additional heterozygous variants of uncertain significance found in this study. These variants are unlikely to be pathogenic in the subjects but we were unable to exclude the possibility that they act as modifying alleles. LCA, Leber's congenital Amaurosis;

RP, retinitis pigmentosa; BBS, Bardet Biedl Syndrome; CORD, cone-rod dystrophy; eoRP (early onset RP). Homozygous mutations are indicated by (hom).

subject	DX	Disease causing gene	Other genes	Nucleotide change	Protein change	dbsnp	Citation
JB40	Multiplex RP	unknown	RPGRIP1L	c.685G>A	p.Ala229Thr	rs61747071	[24]
			RPGRIP1L	c.2952G>C	p.Gln984His	rs775144757	
			RPGRIP1L	c.196C>A	p.Gln66Lys	rs751444506	
			CDH23	c.1096G>A	p.Ala366Thr	rs143282422	[91]
			CDH23	c.3293A>G	p.Asn1098Ser	rs41281310	[97]
			FAM161A	c.977A>C	p.Lys326Thr	rs745318331	
			GUCY2D	c.3247C>A	p.Leu1083Met	N/A	
			USH2A	c.6713A>C	p.Glu2238Ala	rs41277212	[77]
			USH2A	c.1434G>C	p.Glu478Asp	rs35730265	[98]

Table 2-5: Multiple compound heterozygous mutations were identified in a subject with multiplex retinitis pigmentosa. This family appears to have an unusually high number of recessive mutations rather than the single dominant mutation that was expected based on the family history. We were unable to perform segregation analysis. The CDH23 mutations and RPGRIP1L mutations are the most likely to be pathogenic.

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Chapter 3: Identification of a novel pathogenic missense mutation in PRPF31 using whole exome sequencing

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Abstract:

Background: Variants in *PRPF31,* which encodes pre-mRNA processing factor 31 homolog, are known to cause autosomal dominant retinitis pigmentosa with incomplete penetrance. However, the majority of mutations cause null alleles, with only two proven pathogenic missense mutations. We identified a novel missense mutation in *PRPF31* in a family with ADRP.

Methods: We performed whole exome sequencing to identify possible pathogenic mutations in the proband of a family with ADRP. We carried out segregation analysis of the probable mutation, *PRPF13* c.590T>C. We evaluated the cellular localization of the PRPF31 variant (p.Leu197Pro) compared to the wildtype PRPF31 protein.

Results: *PRPF31* c.590T>C does segregate with disease in this family. One family member has the mutation but is unaffected, which is consistent with previous reports the incomplete penetrance of PRPF31 mutations. Unlike the wildtype PRPF31 protein, the mutant PRPF31 protein (p.Leu197Pro) does not localize to the nucleus. Instead, it localizes primarily to vesicles in the cytoplasm.

Conclusions: c.590T>C is a novel pathogenic variant in *PRPF31* causing autosomal dominant retinitis pigmentosa with incomplete penetrance likely due to protein misfolding.

Background:

Retinitis pigmentosa (RP) is the most common form of inherited retinal degeneration, affecting 1 in 2500-7000 people[1]. Over 60 genes have been shown to cause RP[2]. It can be inherited in an autosomal recessive, autosomal dominant or x-linked inheritance pattern, with different genes associated with each inheritance pattern[3]. Approximately 30-40% of the cases of retinitis pigmentosa are autosomal dominant[3]. Dominant disease can be due to either a toxic gain of function in which the mutation causes the protein to have a directly toxic effect on the cell or haploinsufficiency, where half the normal amount of protein is insufficient to fulfill the needs of the cell.

PRPF31 encodes a ubiquitously expressed splicing factor[4]. It links the U4/U6 complex with U5, creating the tri-snRNP of the spliceosome[5]. *PRPF31* mutations are known to cause autosomal dominant RP with incomplete penetrance (RP11, OMIM 600138)[4,6-11]. It is interesting that *PRPF31* mutations result in a retina specific phenotype when the splicing factor is ubiquitously expressed. A retina specific isoform has not been identified that can explain the tissue specific susceptibility of the retina to a heterozygous mutation in *PRPF31*[12]. The retina appears to simply have a higher dependence on this splicing factor than other tissues. Deery et al speculated this could be due to the need to constantly replenish disc proteins in the outer segments, resulting in a higher splicing load than in other cell types[13]. It is also possible that there are splicing factors in other cell types that can compensate for the reduced PRPF31 levels that are not expressed in the retina.

The incomplete penetrance seen in families with *PRPF31* mutations is due to the variable expression levels of *PRPF31*[9,10]. There is a critical level of PRPF31 needed to avoid retinal degeneration. If both alleles are wildtype, the critical level is exceeded and retinal degeneration is avoided. However, if the expression level of one wildtype allele is high enough, a carrier of a pathogenic allele will still reach the critical level of wildtype protein and be asymptomatic. If the wildtype allele has an average or low level of expression, a carrier of a pathogenic allele will develop retinal degeneration.

We identified a novel missense mutation in *PRPF31*. Most mutation in *PRPF31* are truncations, deletions or frameshift mutations which result in a null allele[14]. However, two missense mutations have been shown to be pathogenic and are located within 20 amino acids of the novel mutation seen in the family in this study, suggesting it could be in an important domain for protein function. We analyzed the segregation of the mutation within the family and the impact of the mutation on localization of the protein and concluded that it was the disease causing mutation in this family.

Methods:

Whole Exome Sequencing: Testing was carried out samples from human subjects after obtaining written informed consent on an Institutional Review Board (IRB) approved protocol (#808828). A sample from the proband was screened previously for mutations in rhodopsin, peripherin/RDS and ROM1 (Carver Lab, University of Iowa, Iowa City, IA, 1995) and found to be negative. For whole exome sequencing, we performed target enrichment using Agilent SureSelect target enrichment system and whole exome

sequencing was performed on Illumina HiSeq2000 at the Penn Genome Frontiers Institute (PGFI). We used Galaxy to analyze the whole exome sequencing data set[15]. The FASTQ files were aligned to hg18 using BWA for Ilumina (Burrow-Wheeler Aligner). Variants were identified with Freebayes and the variants were annotated using Annovar[16,17]. We then filtered the variants to include only those in genes linked to retinal degeneration that had an allele frequency of 0.05 or less. This yielded a short list of genes which were prioritized based on mutation type and likelihood to cause autosomal dominant retinitis pigmentosa. The *PRPF31* variant was the strongest candidate.

Segregation Analysis: We used PCR to amplify the DNA region that included the variant from the proband and all relatives who provided DNA using Phusion (New England Biolabs, Ipswich, MA) and the following primers synthesized by Invitrogen (Carlsbad, CA): GAGCCTTCCTGAGTTCCCG and GCCAAAGCCCCCATTCTAC. The PCR product was sent for sanger sequencing at the Penn Genomics Analysis Core and visualized using SnapGene software (from GSL Biotech (Chicago, IL); available at snapgene.com).

Cloning: We cloned the sequences for PRPF31 from a cDNA library generated from 293T cells. We used Q5 polymerase (New England Biolabs) and custom primers synthesized by Invitrogen. The PCR product was TOPO cloned and the sequences were verified by Sanger sequencing at the Penn Genomics Analysis Core. The coding sequences were cloned into an expression vector with an HA tag using In-Fusion (Takara Bio, Mountain View, CA). The expression vector used the CMV/CBA promoter to drive expression.

Mutagenesis: We used site directed mutagenesis (QuikChange II Site-Directed Mutagenesis Kit from Agilent Technologies; Santa Clara, CA) to introduce the p.L197P variant into the cloned *PRPF31* using the following primers: GATGCGGTGCTTGGAGGCGTTCGGCTCCAGCGCCATGTCGCAG and CTGCGACATGGCGCTGGAGCCGAACGCCTCCAAGCACCGCATC.

Transfection: ARPE19 cells were maintained in DMEM F12 media (Invitrogen) supplemented with 10% fetal bovine serum. We plated the cells in 4 well chamber slides. The cells were transfected using Lipofectamine LTX with plus reagent (Thermo Fisher Scientific; Agawam, MA) and fixed 48 hours post-transfection.

Immunofluorescent staining: The ARPE19 cells were fixed for 15 minutes in 4% paraformaldehyde. They were rinsed three times with Dulbecco's phosphase-buffered saline (Corning 21-030-CV). We then blocked the cells for one hour using a blocking buffer consisting of 10% normal goat serum and 0.5% Triton X-100 in PBS. The slides were then incubated overnight at 4 degrees with an antibody against the HA tag (Cell Signaling Technology #3724; Danvers, MA) at a 1:800 dilution. The slides were then incubated at room temperature for 3 hours in Alexafluor 488 Goat anti-Rabbit (ThermoFisher Scientific A-11034). The cells were mounted using Fluoromount-G mounting media contained 4',6-Diamidino-2-Phenylindole (DAPI). The cells were imaged using an Olympus FV1000 confocal microscope with a 60X oil immersion objective.

Results:

A 37 yo woman presented with worsening vision and light sensitivity over the preceding 5 years. Previous electroretinogram (ERG) testing had shown near normal cone responses with severe rod B wave amplitude loss. Her brother had also complained of worsening vision. Clinical examination showed visual acuity of 20/25 in the right eye and 20/30 in the left eye. The retinal examination showed marked vessel attenuation, waxy pallor of the optic disc, and relatively symmetrical mid-peripheral pigment epithelial atrophy (Figure 3-1A). There were neither bone spicules nor vitreous debris typical of retinitis pigmentosa. Visual field testing showed an equatorial scotoma in the temporal retinas of both eyes (Figure 3-1B). The family history was consistent with an autosomal dominant pattern of inheritance with incomplete penetrance (see Figure 3-1C). To her memory, her father had not received a diagnosis with any form of retinal degeneration. Two of her brothers had been diagnosed with retinitis pigmentosa (Figure 3-1C). None of the proband's children (current ages 26, 30 and 32) have been diagnosed with retinal degeneration.

Analysis of the whole exome sequencing data shows that the proband has a novel c.590T>C (p.Leu197Pro) missense variant in *PRPF31* (Figure 3-1D). This variant is not listed in dbSNP or ExAC. Two missense mutations in *PRPF31* that are known to be pathogenic are p.Ala194Glu and p.Ala216Pro. The proximity of the new variant with these other amino acid changes suggests that this region is an important domain for protein function[11]. Since PRPF31 is a splicing factor, mislocalization from the nucleus would essentially constitute a loss of function mutation. Both the p.Ala194Glu and

p.Leu197Pro variants are located in the second coiled coil domain while the p.Ala216Pro links the coiled coil to the Nop domain which is a ribonuclearprotein (RNP) recognition motif[18].

Three of the proband's siblings (one affected, two unaffected) were tested for the c.590T>C mutation in *PRPF31* (see figure 3-1C). One affected brother was heterozygous for the variant while two of the unaffected siblings were homozygous for the wildtype allele. One of the unaffected siblings (sister, II-4) is a carrier for the c.590T>C variant. In sum, the inheritance pattern is consistent with AD disease with incomplete penetrance typically seen in *PRPF31* mutations. Although the asymptomatic carrier may continue to enjoy good vision, any children that she may have who inherit the mutation will be at risk for retinal degeneration.

Since the previously known *PRPF31* missense variant proteins mislocalize to the cytoplasm[13], we tested the cellular localization of the p.Leu197Pro variant. We cloned the cDNA sequence for PRPF31 from 293t cells and verified that it matched the reference sequence. We then performed site directed mutagenesis to add the c.590T>C variant. We cloned both $PRPF31^{WT}$ and $PRPF31^{L197P}$ into expression vectors, each with driven by a constitutive promoter (the chicken b actin promoter and cytomegalovirus enhancer (CMV/CBA)) and the transgene was tagged with the human influenza hemagglutinin (HA) marker. We transfected ARPE19 cells and analyzed protein localization 48 hours post transfection with confocal microscopy. $\mathrm{PRPF31}^{\mathrm{WT}}$ localized almost exclusively to the nucleus with a small amount of protein seen in the cytoplasm in cells with particularly high expression (Figure 3-2). In contrast, the $\mathrm{PRPF31}^{\mathrm{L197P}}$ variant had a vesicular

staining pattern in the cytoplasm, possibly indicating that it is being targeted for degradation.

Discussion:

Mutations in *PRPF31* are well known to cause ADRP with incomplete penetrance. Most of these mutations are truncations, frameshifts, splicing mutations or large deletions, all of which cause null alleles. Pathogenic missense mutations in *PRPF31* are much rarer. The universal protein resource site (uniprot.org) only lists two missense mutations associated with RP11. The single nucleotide polymorphism database (DbSNP) only classifies two missense variants in *PRPF31* as "pathogenic", while listing three more as "likely pathogenic". Given that the RP11 phenotype involves incomplete penetrance and pathogenic missense variants are rare, functional testing is more important than usual to establish pathogenicity. The fact that the variant PRPF31 is not localized to the nucleus makes it impossible for PRPF31^{L197P} to be functional as a splicing factor.

The mislocalization seen in the PRPH31 p.Leu197Pro protein variant as well as the previously described p.Ala194Glu and p.Ala216Pro variants is interesting considering they are unlikely to disrupt a nuclear localization signal. Ala194 and Leu197 are located in a coiled coil domain while Ala216 is in a linker region between the coiled coil and the NOP domain[18]. It seems likely that these missense mutations are destabilizing the protein structure or disrupting protein folding and causing the protein to be targeted for degradation. This is consistent with the staining pattern we observed for PRPF31^{L197P}.

Conclusions:

We conclude that the c.590T>C missense variant in *PRPF31* is a pathogenic mutation causing autosomal dominant retinitis pigmentosa with incomplete penetrance. The phenotype is consistent with the phenotype seen from other *PRPF31* mutations, the mutation segregates with disease in the family with incomplete penetrance, and the missense mutation causes mislocalization of the protein *in vitro*.

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Figures:

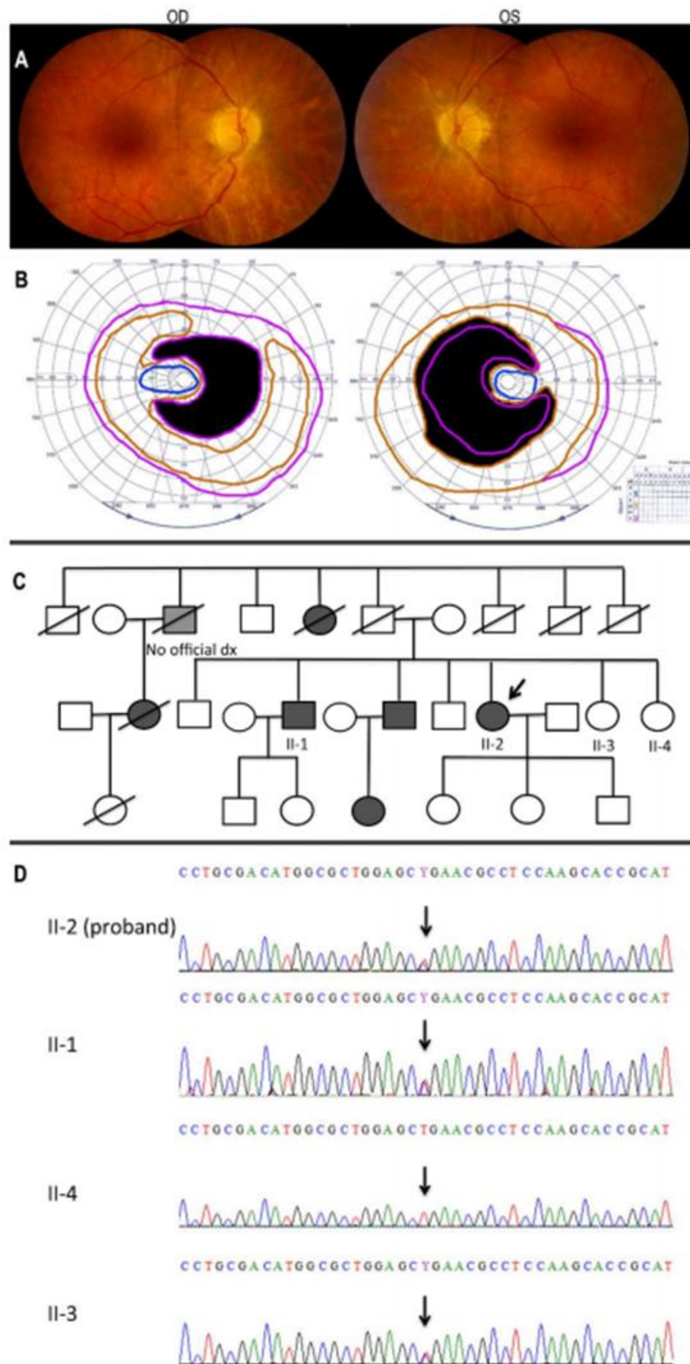

Figure 3-1: Clinical features and molecular data for *PRPF31* family. A) Fundus photos of the proband taken on her first visit (age 37). There is marked vessel attenuation, waxy

pallor of the optic disc, and relatively symmetrical mid-peripheral pigment epithelial atrophy. OD, right eye (ocula dextra); OS, left eye (ocula sinestra); B) Visual field testing results of the proband. There are bilateral equatorial scotomas in the temporal retinas; C) Pedigree for the family, which has affected members in three generations, both male and female. The father of the proband was apparently unaffected, indicating incomplete penetrance. DNA samples were obtained from the numbered family members. The proband is indicated with an arrow; D) .Sanger sequencing results for the *PRPF31* c.590T>C variant. The variant was confirmed in the proband (II-2) and the affected brother (II-1). One of the unaffected sisters (II-4) is homozygous for the wildtype allele while the second unaffected sister is a carrier for the variant (II-3).

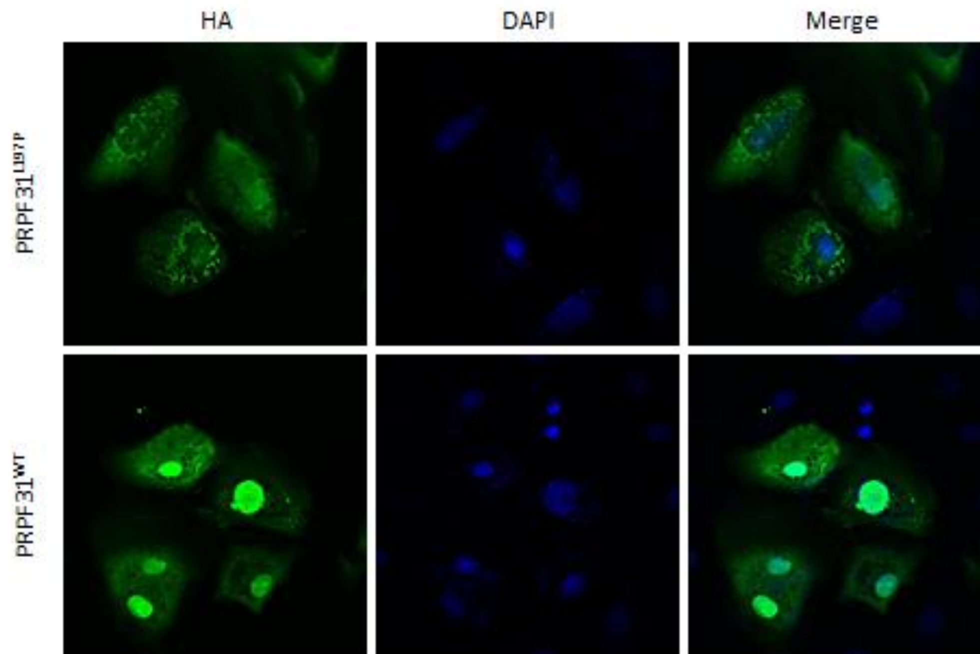

Figure 3-2: Localization of PRPF31^{WT} and PRPF31^{L197P} in ARPE19 cells as measured by immunofluorescence for the HA tag (green). Nuclei appear blue due to staining with 4',6-Diamidino-2-Phenylindole (DAPI). PRPF31^{L197P} mislocalized to the cytoplasm while PRPF31^{WT} localized primarily to the nucleus. Note the vesicular staining pattern for the PRPF31^{L197P} construct.

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Chapter 4: On Variants and Disease-Causing Mutations: Case Studies of a SEMA4A Variant Identified in Inherited Blindness

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Three different missense variants in *SEMA4A* have been identified in retinitis pigmentosa (RP) patients and presumed to be pathogenic: p.R354H, p.F350C and p.R713Q[1]. p.R354H and p.F330C were reported to be recessive mutations while p.R713Q was reported to be a dominant mutation. The variants were classified as pathogenic since: 1) they segregated with the disease in the respective families, and 2) they were not found in 100 ethnically matched normal-sighted control individuals[1]. Here we review laboratory results relating to *SEMA4A* variants and present data that contradict previous conclusions that particular *SEMA4A* variants are pathogenic.

Nogima et al created knock-in mouse lines for *SemA4a* missense variant[2]. Of the three variants, only the p.F350C variant resulted in retinal degeneration in mice[2]. As the authors mention, it is possible that the difference in *SEMA4A* sequence between human and mouse can account for the differences in the effects of the variants between these two species. However, it is also possible that the variant is not pathogenic and is merely a benign polymorphism or a risk factor for blindness that is not sufficient to cause disease on its own. Additional studies in the ARPE19 human retinal pigmented epithelium (RPE) cell line demonstrated that the p.D345H and p.F350C variants do not properly localize to the cell membrane and also cause deficits in phagocytosis or ER stress response to oxidative stress[3]. Conversely, the p.R713Q variant did not affect phagocytosis, ER stress response or protein localization[3].

After obtaining consent and collecting blood samples for DNA (UPenn IRB #808828), we analyzed whole exome sequencing data from patients with genetic forms of retinal degeneration seen at the Scheie Eye Institute Department of Ophthalmology. We discovered three unrelated subjects who were heterozygous or homozygous for the

p.R713Q variant of *SEMA4A*. Further analyses showed that the variant did not segregate with the disease in any of the families. The details are as follows:

Family A: This family has autosomal dominant retinal degeneration which is manifest as retinitis pigmentosa in some individuals (II-2) and macular dystrophy in others (II-4, III-1, II-1). The p.R46X mutation in *PRPH2*, known to be pathogenic, segregates with the disease. The proband is a 74 year old woman with macular dystrophy (II-4, Figure 4-1A) who has been followed for the past 39 years. While most of the relatives tested had retinal degeneration and were heterozygous for the p.R713Q variant in *SEMA4A*, the brother of the proband, II-7, was heterozygous for the *SEMA4A* variant (but not the *PRPH2* mutation) and had no symptoms or signs of retinal/macular degeneration.

Family B: A 43yo female (proband II-2, Figure 4-1B) presented with unilateral pigmentary retinal degeneration. She had been symptomatic since age 19. Examination was notable for marked asymmetry, with bone spicules and peripheral to central retinal degeneration in the right eye only and asymmetric ERGs and visual fields. Over the next 14 years, lattice degenerative changes commenced in the left eye. Neither parent had a history of retinal disease. The proband II-2 was diagnosed with simplex RP. Neither the 30yo son nor the 32yo daughter of the proband shows signs of retinal disease. The proband is homozygous for the p.R713Q variant of *SEMA4A*. The son (III-1) and the daughter (III-2) are heterozygous for the p.R713Q variant of *SEMA4A* . Based on the pedigree of this family, it would be possible for the p.R713Q variant of *SEMA4A* to cause AR disease, but it is not consistent with a dominant mutation. We were unable to positively identify the pathogenic mutation(s) in this proband after exploring numerous potential candidates (including common causes of AR and ADRP as well as *PER3,*

HOXD1, DLEC1, ALS2CL, COL4A1, MRPS31, and *STARD8*). We suspect that either a novel gene is responsible or a *de novo* mutation arose in the one affected retina.

Family C: An otherwise healthy 67yo male (proband II-3, Figure 4-1C) presented with light perception only vision. An ophthalmic exam revealed widespread pigmentary changes, retinal thinning and vessel attenuation. He had been diagnosed with simplex RP in his 20's. His sisters and brothers and two sons (each in their 30's) had normal vision. Genetic testing revealed that the proband is heterozygous for the p.R713Q variant in *SEMA4A*. Three of his unaffected siblings are also heterozygous for the variant. Additionally, his unaffected 43yo son is homozygous for the p.R713Q variant of *SEMA4A*. The fact that an unaffected family member is homozygous for the mutation indicates that this mutation is insufficient to cause disease. The pathogenic mutations in this family is likely to be in *USH2A* as the proband has compound heterozygous mutations in *USH2A* (p.R4192H and p.R1653*) and no other family member has mutations in both alleles.

In summary, we describe three families with retinal degeneration and in which the *SEMA4A* p.R713Q variant was observed in both affected and unaffected individuals. Our findings are inconsistent with the dominant pattern of inheritance currently ascribed to the *SEM4A* p.R713Q variant[1]. Not only is there a lack of segregation of the mutation with disease, but also one of the unaffected family members in family C is homozygous for the variant, thus eliminating the possibility that the variant leads to a recessive disease. These results are consistent with the results from the mouse model generated by Nogima et al that was homozygous for the p.R713Q missense *Sema4a* variant. This mouse did not show any signs of retinal degeneration.[2,3] It is possible that the p.R713Q missense

Sema4a change could lead to disease when combined with a mutation in another gene, but it is not sufficient to cause disease in isolation.

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Figures:

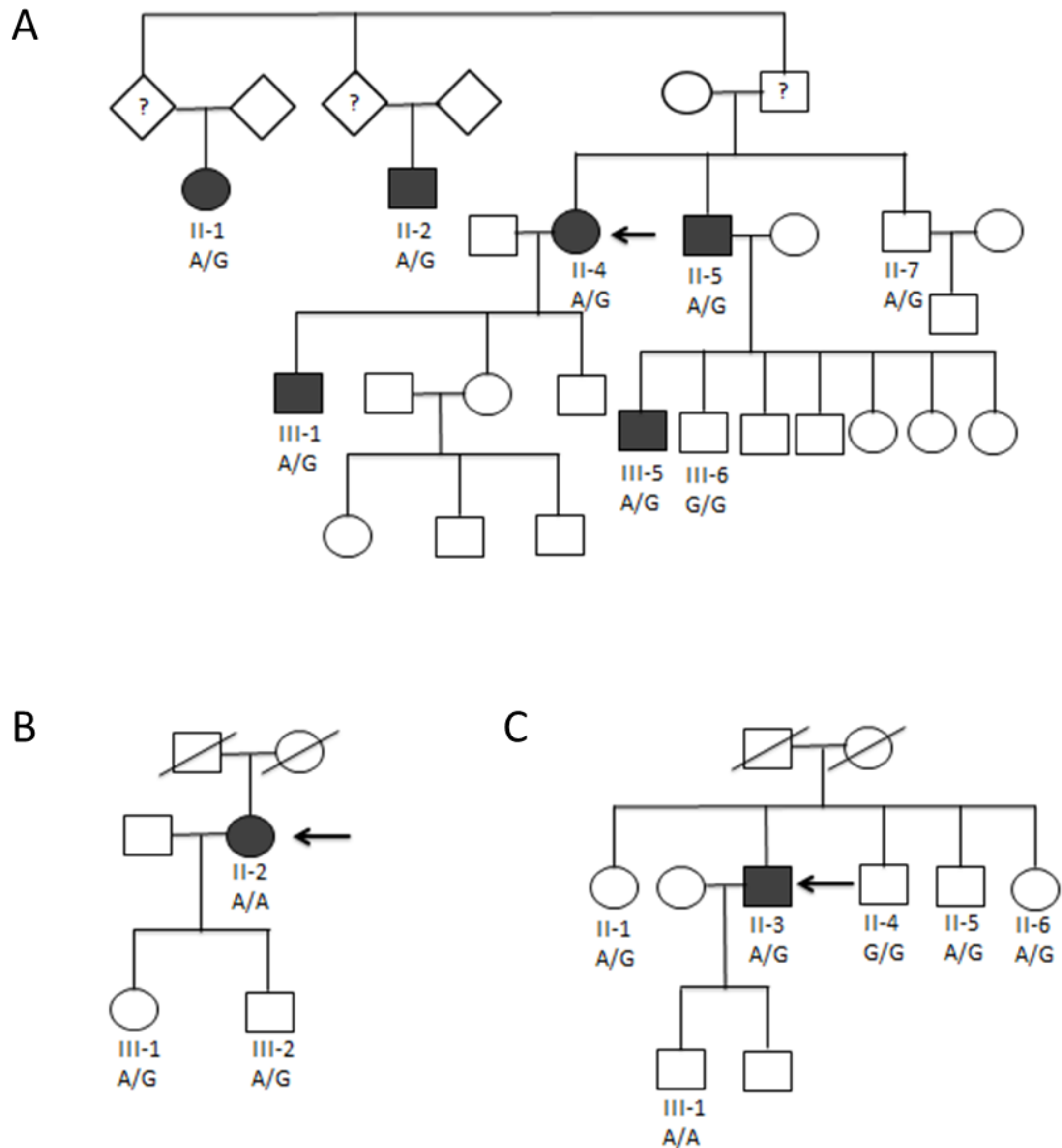

Figure 4-1: Family Pedigrees and clinical findings. Three families carry the p.R713Q missense variant in *SEMA4A* (c.2138G>A). A) Family A has a dominant inheritance pattern with multiple affected individuals. B) Family B has one affected member with autosomal recessive retinitis pigmentosa. C) Family C has one affected member with autosomal recessive retinitis pigmentosa.

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Chapter 5: Loss of ON-bipolar cell specific expression from the GRM6 and TRPM1 promoters during retinal degeneration

Laura Bryant*, Pavitra Ramachandra*, Jean Bennett

*These authors contributed equally to this work.

Modified version submitted to PLOS One

Introduction:

According to the WHO, 39 million people worldwide are totally blind (http://www.who.int/mediacentre/factsheets/fs282/en/). For these patients, very limited or no treatment options exist. In many cases, the underlying causes of retinal blindness are genetic. Gene replacement and gene knockdown strategies aiming to restore vision are being tested and move toward clinical trials for such individuals, but these therapies will only work if the retinal neurons are still present in the patient. Additionally, it would be prohibitively expensive to develop a gene augmentation therapy for each of the >200 different genes that contribute to blinding retinal diseases, some of which have only been identified in a handful of patients worldwide. For many of the advanced stages of retinal degeneration, there are no available treatments or therapies. In these patients, most of the photoreceptor cells (rods and cones) – the light sensors of the eye, are degenerated and/or lost.

Recently, optogenetic strategies to restore light sensitivity to the remaining cells of the retina (photoreceptors, bipolar cells or ganglion cells) have seen considerable success in mice[1-4]. Optogenetics involves the introduction of genetically encoded light sensors to

cells to make them responsive to light. In severe retinal degeneration, targeting these light sensors to retinal cells will create artificial photoreceptors and stimulate the remaining retinal circuitry to function. There are a few different ways by which retinal activity can be restored in the non-functioning retina using optogenetics, but based on what is most well understood, three different strategies have been used in animal models[5]. In one strategy, Busskamp *et al*. genetically targeted a light sensitive chloride channel - enhanced *Natronomonas pharaonis* halorhodopsin (eNpHR), which hyperpolarizes in the presence of light, to the photoreceptors present in blind mice and reported restoration of visual responses and visually guided behavior[1]. In a second strategy, Lagali *et al*. targeted a different light sensor that depolarizes in the presence of light – channelrhodopsin-2 (ChR2) to the ON-bipolar cells of blind mice using an ON bipolar cell (BC) specific promoter[6]. These mice demonstrated an improvement in visually guided behavior and locomotion indicating a restoration of visual function. Engineering of the *ChR2* gene gave rise to a modified version called CatCh with increased sensitivity to light and faster channel kinetics[7].. Another strategy is to target the retinal ganglion cell. Bi *et al* targeted *CHR2* by using a ubiquitous promoter and administering via subretinal injection. While this strategy does result in in ganglion cell responses, it eliminates all retinal processing and activates both ON and OFF pathways simultaneously[8].

ON bipolar cells are particularly attractive targets because they would theoretically retain the most retinal signal processing after photoreceptor cell death. In order to effectively target the ON bipolar cells without also expressing the optogenetic protein in the OFF bipolar cells, the murine metabotropic glutamate receptor 6 (*mGRM6)* promoter has been

used as a cell specific promoter[2,3]. It is expressed in both the ON cone bipolar cells and the rod bipolar cells (which are also ON bipolar cells). Since mGluR6, the protein product of *GRM6,* is involved in the signal inversion necessary for the ON pathway, it is not expressed in the OFF bipolar cells.

The 9.5 kb *mGRM6* promoter was identified by Ueda *et al.* and used to create a transgenic mouse line with β-galactosidase expression in the retina restricted to ON bipolar cells[9]. While the expression profile was very specific, the promotor was too large for AAV, which is currently the viral vector of choice for gene therapy targeting neural retina. An essential 200bp enhancer sequence was identified within the full promoter that conveys bipolar cell specific expression when paired with the ubiquitous SV40 promoter in the wildtype mouse retina[10]. Adding additional copies of the enhancer increased expression levels and specificity, with four copies achieving the optimal expression (referred to as the 4x*GRM6*-SV40 promoter) [3].

Early studies have shown that 4x*GRM6*-SV40 promoter can be used to drive channelrhodopsin expression in ON bipolar cells. They showed specific expression in the ON bipolar cells of the treated wildtype (WT) mice and behavioral improvement in rd1 mice with retinal degeneration[2,3,11]. However, a recent study found that bipolar cell specific expression was not observed using the 4x*GRM6*-SV40 promoter in rd1 mice[12].

We used the 4x*GRM6*-SV40 promoter and the TRPM1 promoter to drive eGFP expression in the ON-bipolar cells of mice with retinal degeneration to test the specificity of the promoters in the degenerate retina. The 4x*GRM6*-SV40 promoter drove low level

expression in bipolar cells, ganglion and amacrine cells. Surprisingly, we occasionally saw expression in the retinal pigmented epithelium (RPE). The TRPM1 promoter also failed to provide ON bipolar cell specific expression in mice with retinal degeneration whereas it drove high levels of ON bipolar expression in mice with wildtype retinas. This dramatic change in expression profile suggests that retinal degeneration has a more profound effect on gene expression than previously suggested and that optogenetic therapy for late stage retinal degeneration may be better targeted to ganglion cells to bypass all retinal processing.

Methods:

Expression constructs: The 4x*GRM6*-SV40-CatCh-eGFP construct was a kind gift from Dr. Botond Roska. It was packaged it in AAV7m8 and AAV8BP2 at the Center for Cellular and Molecular Therapeutics at the Children's Hospital of Philadelphia. For later experiments, we removed the coding sequence for CatCh and used only 4x*GRM6*-SV40-eGFP and that was packaged in AAV8BP2 and AAV7m8 at the CAROT Research Vector Core. The human TRPM1 promoter was cloned from human genomic DNA by PCR amplification (forward primer acgcgtagccactcaccagac; reverse primer ggatccctcctgagtttgtccac) and inserted in front of the CatCh-eGFP transgene. The TRPM1 vectors were packaged into AAV7m8 and AAV8BP2 at the Centre for advanced retinal and ocular therapeutics (CAROT).

Animals and injections: We obtained C57Bl6/J, *rd1* (C3H/HeJ), *rd10* (B6.CXB1-Pde6b$^{rd10/J}$), *rd16* (BXD24/TyJ-Cep290$^{rd16/J}$) and *Lca5 mutant* mice

(*Lca5*$^{Gt(AG0283)Wtsi/\ Gt(AG0283)Wtsi}$) from Jackson laboratory and maintained them under a 12 hour light dark cycle (see table 5-1 for details on mouse lines). Mice were injected with ~ 3 uL of virus either subretinally or intravitreally under isofluorane anesthesia as previously described[13]. Both AAV7m9-4x*GRM6*-SV40-CatCh-eGFP and AAV8BP2-4x*GRM6*-SV40-CatCh-eGFP were injected at a titer of 1E+13vg/ml and AAV8b-4x*GRM6*-SV40-eGFP was injected at a titer of 9.48E+12vg/ml. The *rd1* mice were injected at 1 month and 8 months of age. *Rd16* and *rd10* mice were treated at 5 months of age at the *LCA5* mice were treated at 6 months. Eyes were harvested 4-8 weeks post injection and fixed in 4% paraformaldehyde for 20 minutes. The eyes were frozen and cryosectioned. For the TRPM1 studies, mice were injected with ~3ul subretinally (AAV8BP2) or intravitreally (AAV7m8) – TRPM1-CatCh-eGFP at a titer of 8.19E+12vg/ml. Wildtype mice were injected at 1 month of age and eyes were harvested one month post injection. Rd1 mice were injected at 4 weeks of age and eyes were harvested at 4 months post injection.

Retinal explant cultures: Retinas were isolated from a 5 year old Crd1 dog with advanced retinal degeneration post euthanasia and cultured as described previously (Cronin et al.) for 12 days. They were then mounted on slides and imaged using the Olympus FV1000 confocal microscope with a 40X oil immersion objective.

Immunohistochemistry: The retinal sections were blocked with a blocking buffer consisting of 10% normal goat serum and 0.5% Triton X-100 in Phosphate Buffered Saline (PBS). We incubated the slides overnight with a rabbit anti-GFP antibody (Thermofisher scientific #A-11122) at a 1:300 dilution. We then incubated the slides for

three hours at room temperature with Alexafluor 488 Goat anti-Rabbit (ThermoFisher Scientific #A-11034). We mounted the coverslips using Fluoromount-G mounting media contained DAPI. We imaged the slides using an Olympus FV1000 confocal microscope with a 40X and 60X oil immersion objective or with a Zeiss fluorescent microscope.

Results:

To compare transduction efficiency of the 4XGRM6-SV40 promoter in bipolar cells of healthy and degenerating retinas, we used two different AAV serotypes- AAV7m8 and AAV8BP2. AAV8BP2 has been shown to target the bipolar cells efficiently in wildtype mouse retinas and to elicit ganglion cell responses in the *rd1* mice (Cronin et al). AAV7m8 can also target the bipolar cells by subretinal and intravitreal routes of injection and has been used to target ChR2 to the ON-bipolar cells for optogenetic therapy in an RP mouse model[11]. In this study, we administered AAV8BP2 or AAV7m8 containing the 4x*GRM6*-SV40-CatCh-eGFP transgene cassette by subretinal injection in wildtype (WT) and rd1 mice at P90. While CatCh-eGFP expression in the wildtype mouse retinas was robust and restricted mostly to the ON bipolar cells, expression in the *rd1* mice was poor and not bipolar cell specific. Rather, CatCh-eGFP expression seemed to be primarily in amacrine and ganglion cells (Figure 1). Furthermore, no improvement in visually guided behavior (water maze) was observed compared to untreated *rd1* mice (data not shown).

We wondered whether the misexpression of 4x *GRM6*-SV40 promoter in the *rd1* mice was due to the degree of retinal degeneration or if the poor expression in the bipolar cells

was due to the ectopic expression of CatCh. To eliminate these confounding factors we performed further expression studies using the 4x *GRM6*-SV40 promoter driving eGFP expression without CatCh. We injected 1 month old and 8 month old *rd1* mice with AAV8BP2- 4x*GRM6*-SV40-eGFP by subretinal injection and harvested the eyes 6 weeks later. Both the young and old rd1 mice showed non-specific eGFP expression in ganglion cells, muller glia cells and amacrine cells, but the younger mice did show a higher level of expression in more bipolar cells than was seen in the older mice (Figure 2). Although we saw more specific eGFP expression in the younger *rd1* mice, expression in the injected region was patchy, with some areas of the retina showing exclusively bipolar cell specific expression (Figure 3a-d), other areas having bipolar cell and non-specific expression (Figure 3e-h and figure 3i-l), and still others showing no bipolar cell expression at all but expression in a few amacrine or ganglion cells (Figure 3m-p). Islands of remnant cone cell bodies seemed to be associated with areas where the expression was exclusively in bipolar cells, suggesting that the loss of specificity is a result of progressing degeneration. We also occasionally observed eGFP expression in the RPE cells (data not shown).

Rd1 mice are a fast degenerating mouse model of retinitis pigmentosa where remodeling of the inner retinal cells takes place early causing changes in the expression of transcription factors, which could account for the loss of specific expression with the 4x GRM6-SV40 promoter[14,15]. To address this concern, we tested the AAV8BP2 4xGRM6-SV40-eGFP vector in different retinal degeneration mouse models (*rd10*, *rd16* and *LCA5*) having slower rates of retinal degeneration (Table 5-1). We observed poor overall

eGFP expression (even with anti-GFP antibodies) in each of the strains of mice tested. Islands of eGFP expression were seen in each mouse line with bipolar cell expression observed along with expression in ganglion and amacrine cells (Figure 4). Especially strong expression was seen in ganglion cells in the *rd10* line (Figure 4e-h). Overall, we observed a general misregulation of the 4x*GRM6*-SV40 promoter in four retinal degeneration mouse models that results in non-specific expression.

It is also possible that the misregulation of gene expression is limited to the 4x*GRM6*-SV40 promoter and to address this, we tested another bipolar cell specific promoter, TRPM1. Interestingly, TRPM1 expression is retained in the bipolar cells even after photoreceptor loss in the *rd1* mice (Krizaj et al 2010). We cloned the human TRPM1 promoter in front of CatCh-eGFP, packaged it into AAV7m8 and AAV8P2 and injected WT and *rd1* mice between 3 and 4 weeks of age. In wildtype retinas we observed ON-bipolar cell specific expression with both AAV8BP2 and AAV7m8 (Figure 5a,b). We also observed expression in some cells of the outer nuclear layer and RPE with AAV7m8. In *rd1* mice, however, AAV8BP2 only expressed eGFP in the RPE cells (Figure 5c), while with AAV7m8, a similar expression pattern in the inner retina as with the 4x*GRM6*-SV40 promoter was seen, with eGFP expressed in amacrine and few ganglion cells (Figure 5d). As with the 4x*GRM6*-SV40 promoter studies, we did not observe improvements in navigational vision (data not shown).

To understand if these findings were species specific, we tested AAV7m8-TRPM1-CatCh-eGFP in retinal explants of a Crd1 dog model of retinitis pigmentosa[16]. We found a few eGFP positive cells that co-localized with the ON-bipolar cell marker GOalpha

(Figure 5e). We also observed eGFP positive axon terminals in the inner plexiform layer. In previous studies we showed that the AAV8BP2-4XGRM6-SV40 promoter did drive expression in the ON-bipolar cells of the Crd1 dog post-injection but wasn't specific to those cells (Ramachandran et al., 2016).

In order to determine if we could target the ON-bipolar cells with a ubiquitous promoter more efficiently, we administered subretinal and intravitreal injections of AAV8BP2 and AAV7m8 with a ubiquitous (CMV/CBA) promoter driving expression of eGFP in *rd1* mice at six months of age. Although the transduction efficiency was lower than previously seen in WT mice (data not shown), the viruses were still capable of transducing bipolar cells and more efficiently with AAV8BP2 when compared to AAV7m8 (Figure 6 a-d). These studies suggest that while we can transduce the ON-bipolar cells with a ubiquitous promoter in the degenerate retina, we may not be able to restore vision using optogenetic therapy if it requires cell specific expression.

Discussion:

Our studies show that the expression levels and expression pattern of the *GRM6* and *TRPM1* promoters are significantly altered in remaining retinal cells after photoreceptors have degenerated compared to that seen in wildtype retina. This suggests that selecting promoters for optogenetic therapy based on expression patterns in a healthy retina is not the best strategy. A more thorough understanding of gene expression in late stage retinal degeneration would allow for more informed selection of cell specific promoters. The fact that previous studies were able to restore visually guided behavior in blind mice

using the *GRM6* promoter suggests that non-specific, low level expression is sufficient for visual function or that earlier stages of retinal degeneration retain enough *GRM6* promoter activity to allow for some retention of cell specific expression. Further exploration of cell specific promoters in a degenerate retina could result in even better greater visual improvement after optogenetic therapy.

A simplistic assumption is that after photoreceptor degeneration, the inner retina only experienced mild changes and remained, in essence, the same structurally and functionally as the inner retina of a healthy retina and simply lacked signal input. This would be ideal for optogenetic therapy. Simply add the signal at the appropriate place and it will be treated the same as the signal in a non-degenerate retina (minus any retinal processing that would have taken place at an earlier step). This assumption has been disproven. The inner retina only undergoes subtle changes during the early stages of retinal degeneration, but late stage retinal degeneration involves glial hypertrophy, cell migration, and ectopic synapse formation[17-24]. These changes have the potential to drastically affect the utility of optogenetic therapy, especially cell specific optogenetic therapy.

Glasauer *et al* created a transgenic zebrafish that showed expression of a *GRM6* paralog not only in bipolar cells but also in a subset of ganglion and amacrine cells[25]. It is possible that the nonspecific expression we see is due primarily to down regulation in the bipolar cells rather than up regulation in ganglion and amacrine cells. However, the change in the expression profile of mGluR6 in advanced retinal degeneration has been seen in other studies[12]. Van Wyk *et al* observed nonspecific expression from the

4xGRM6-SV40 promoter in rd1 mice, seeing even less bipolar cell expression than we saw, likely due to the fact that they administered the virus intravitreally while we used subretinal injections for AAV8BP2[12]. Tehrani et al observed that mGluR6 was expressed in a subset of ganglion cells in rats following axotomy[26]. It is also expressed in ganglion cells during development[26]. This suggests that the retina deprived of input from the photoreceptors, may revert to a pseudo-developmental state.

With the TRPM1 promoter, the results were surprising given the data from previous studies that endogenous TRPM1 expression is still cell specific and expression levels don't change in *rd1* mice[27]. It is possible that strain background differences or that exogenous promoter expression is modulated in these mice in retinal degeneration.

The CMV promoter appears to be much more promising for optogenetic therapy in the retina. Although it is non-specific, we were able to easily observe expression in the retina while both the *GRM6* and *TRPM1* expression appeared to be very low and required amplification with a GFP antibody to visualize. Intravitreal injections can be used to largely limit the expression to ganglion cells. Further studies will have to be done to determine whether a non-specific strategy is viable for vision restoration.

Overall, it appears that the inner retina undergoes a substantial change in expression profiles that we do not yet understand. Some studies have been done to look at the changes in gene expression patterns in the retina using microarrays[28,29]. Those studies are a good first step towards evaluating the changes that occur in retinal remodeling, but they do have a few major caveats. First, they use pooled cDNA from the entire retina.

Comparing gene expression in a retina with and without photoreceptors will inherently show up regulation of inner retina genes and down regulation of photoreceptor genes simply because the photoreceptor population is either absent or greatly reduced. In order to achieve a true comparison, photoreceptors would either have to be sorted out (possibly using fluorescence-activated cell sorting) or through single cell analysis. The second major drawback of using pooled cDNA is that it will only show changes in the average gene expression and would not be able to determine if some cell types up regulate a gene while other cell types down regulate the same gene. Part of what we saw was that the 4x*GRM6-SV40* promoter appeared to be down regulated in the bipolar cells and up regulated in amacrine and ganglion cells. Another study was done using in situ hybridization in *rd1* and WT mice to compare expression levels of 169 genes[30]. This study provides better insight into the changes in the retina and can eliminate the confounding factors inherent in using pooled cDNA. However, they can only evaluate one transcript at a time and cannot conclusively identify the identity of the inner nuclear layer cells. Considering late stage retinal degeneration involves cellular migration, identification based on localization is especially risky. Evaluating one transcript at a time eliminates the possibility of evaluating the concomitant up and down regulation of multiple transcripts within the same cell.

More in depth study of the expression profiles of cells in the degenerate retina would help us to not only target specific cell types but also to better understand the cellular responses to photoreceptor degeneration. It is possible that the4xGRM6-SV40 promoter is being misexpressed while the innate *GRM6* promoter is less affected. Further study, including

in situ hybridization in *rd1* mice at early and late stages of retinal degeneration could clarify whether there is up regulation of mGluR6 in other cell types. Most importantly, selection of any cell specific promoters for use in a degenerate retina should be done in a retinal degeneration model as opposed to WT retina.

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Figures:

strain	gene	mutation	Beginning of retinal degeneration	ONL at P30	Rod loss	Cone loss
rd1	*PDE6B*	p.Tyr347X and retroviral insertion in the first intron	P8 (during retinal development)	1 layer	Complete by P30	Complete by P120
rd10	*PDE6B*	p.Arg560Cys	P18	2-3 layers	Complete by P45	P45 has single layer, few remnant cone cell bodies persist through 9 months
rd16	*CEP290*	deletion of exons 35 to 39 (deleted aa1599-1897)	P14	1 layer	Complete by P30	Complete by P90
lca5	*LCA5*	inactivating gene trap insertion in intron 3	P12	3 layers	complete by P60	Complete by P120

Table 5-1: Specific mutations and course of retinal degeneration in mouse lines used in this study.

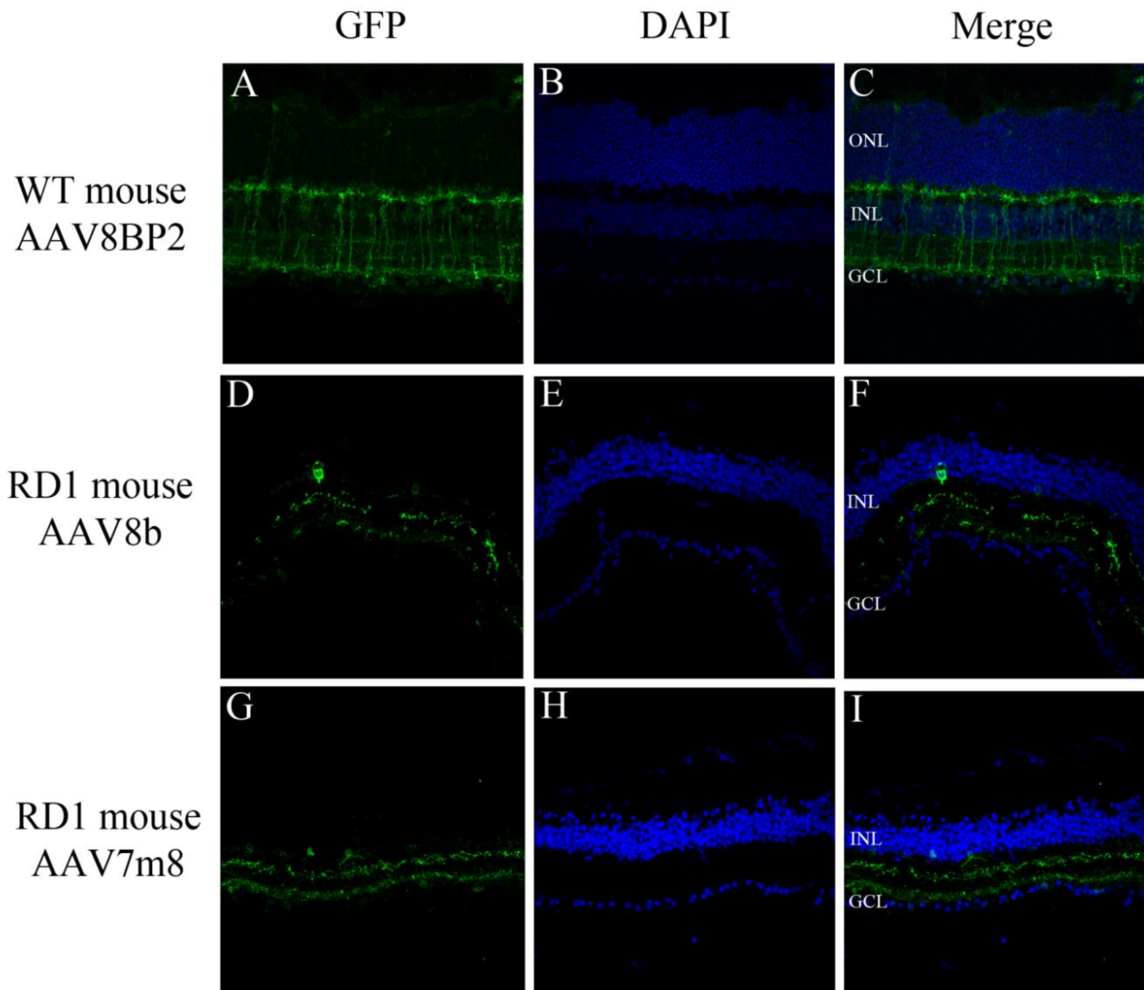

Figure 5-1: Expression profile of the 4x*GRM6*-SV40-CaTCH-eGFP construct in WT and rd1 mice. Note the strong and specific expression in the WT mice in contrast to the sparse amacrine cell expression in the rd1 mice. A-C: WT mouse injected with AAV8BP2-4x*GRM6*-SV40-CaTCH-eGFP. D-F: *Rd1* mouse injected with AAV8BP2-4xGRM6-SV40-CaTCH-eGFP. G-I: *Rd1* mouse injected with AAV7m8-4xGRM6-SV40-CaTCH-EGFP. EGFP expression is seen in green and DAPI staining of the nucleus is in blue. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer

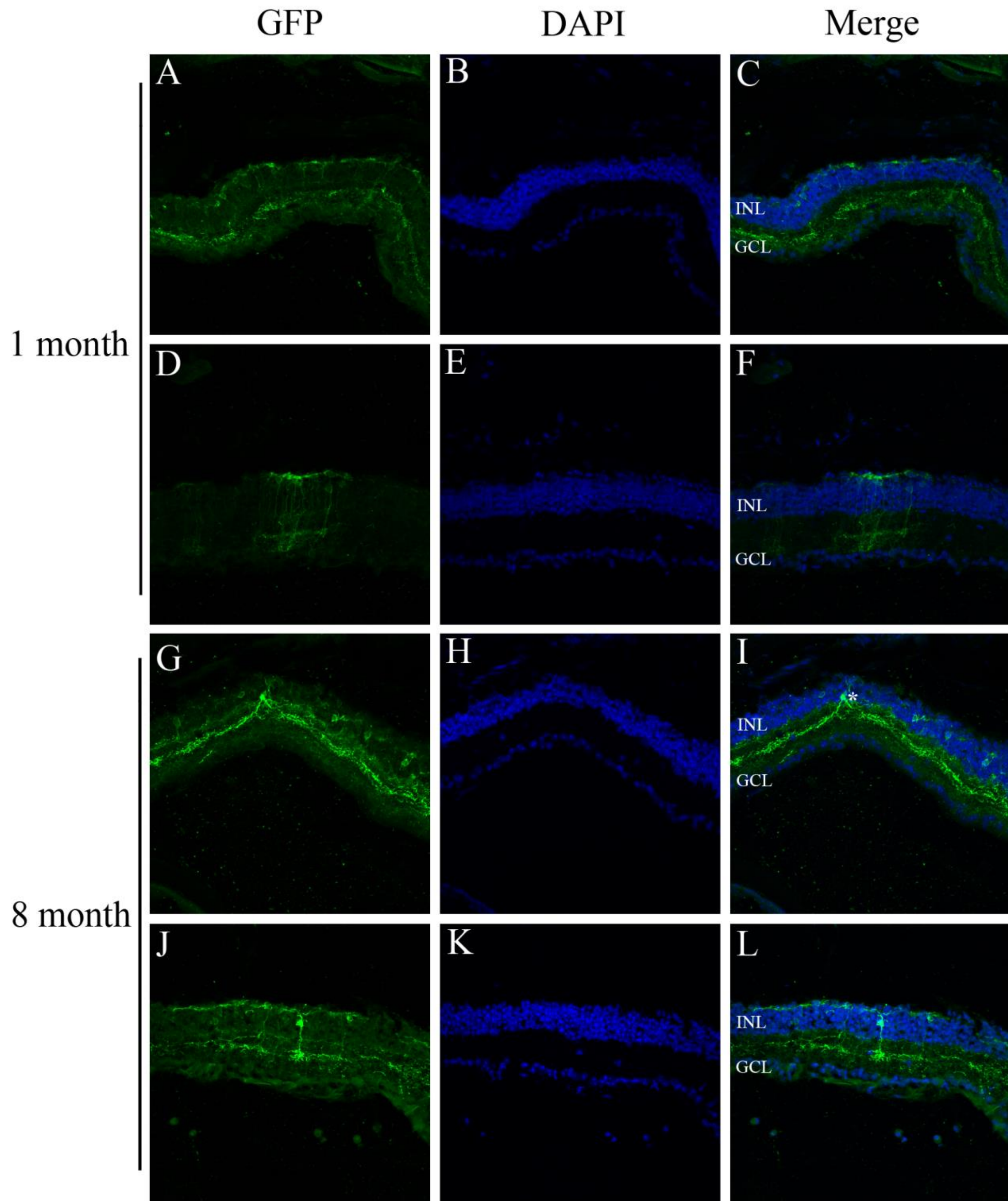

Figure 5-2: Comparison of young (injected at 1 month) and old (injected at 8 months) rd1 mice 6 weeks after injection with the 4x*GRM6*-SV40- EGFP in AAV8BP2. GFP fluorescence is amplified by an anti-GFP antibody. Note the primarily bipolar cell expression in the younger mice with more expression in the amacrine cells (white astrisks) in the older mice. A-C and D-F: 1 month old *rd1* mice injected with AAV8BP2-4xGRM6-SV40-eGFP. G-I and J-L: 8 month old rd1 mice injected with AAV8BP2-4xGRM6-SV40-eGFP. EGFP expression is seen in green and DAPI staining

of the nucleus is in blue. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer.

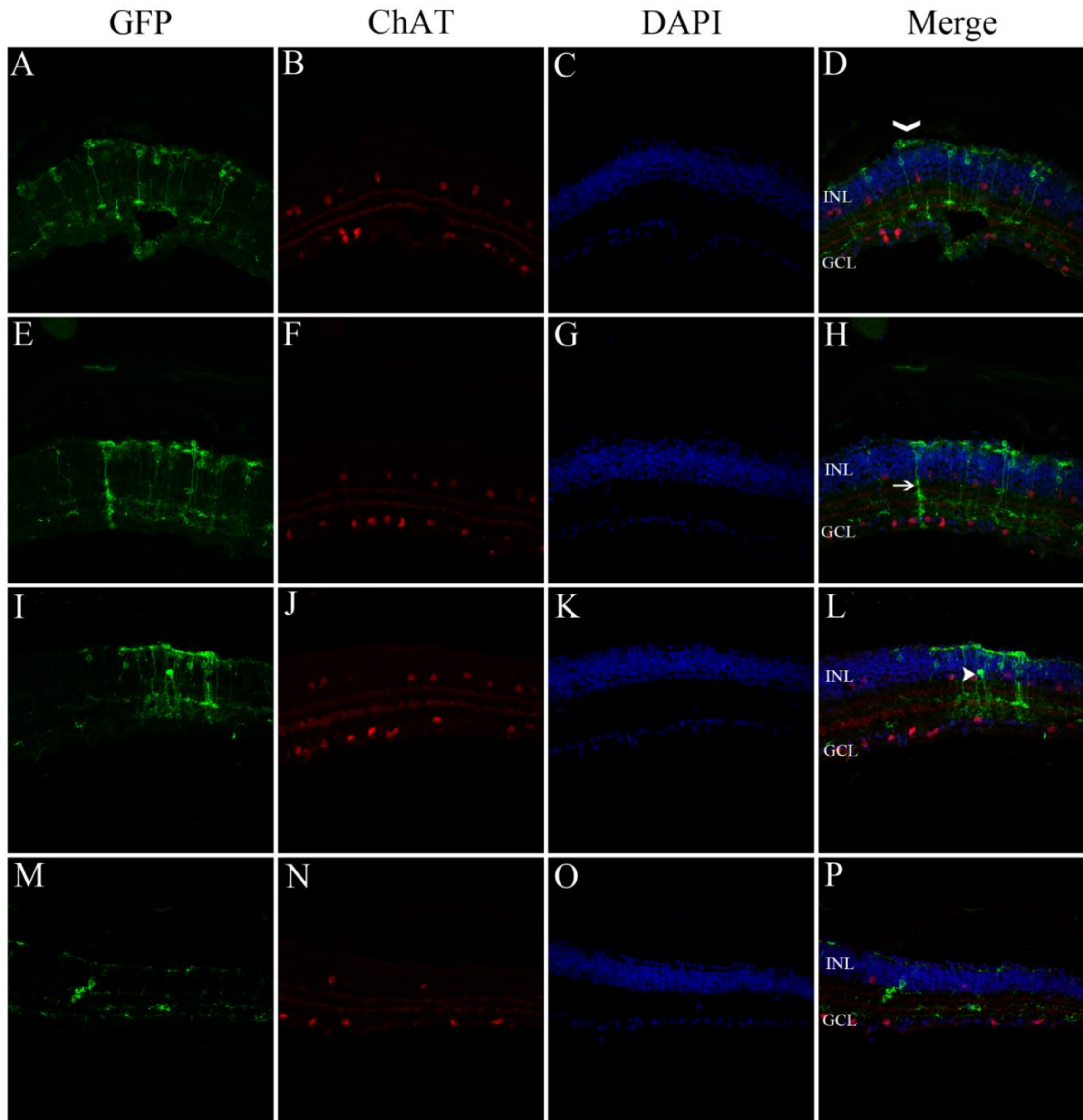

Figure 5-3: Variable expression is seen in the neural retina in different sections within the same eye. All three sections are from a 1 month old rd1 mouse injected with 4x*GRM6*-SV40- EGFP in AAV8BP2. Each row is a different section from the same eye. GFP fluorescence is amplified by an anti-GFP antibody and choline acetyltransferase (CHAT) staining was performed to identify ON and OFF sublamina in the inner plexiform layer. While some areas have primarily bipolar cell expression, other areas have expression in amacrine and muller glia cells. Note the remnant photoreceptors in the top image(D, arrow head), the transduced muller glia cell in the middle

row (H, white arrow) and the transduced amacrine cell (L, white arrow head). EGFP expression is seen in green, ChAT is seen in red and DAPI staining of the nucleus is in blue. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer

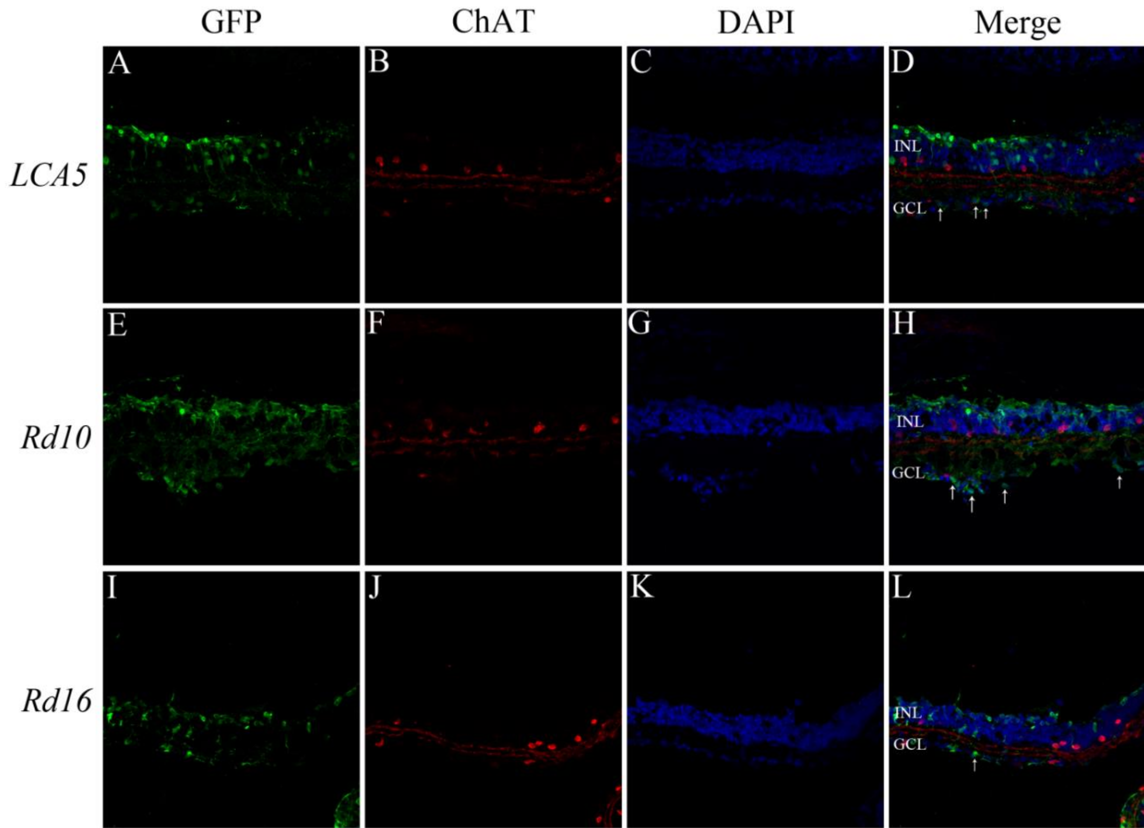

Figure 5-4: 4x*GRM6*-SV40- EGFP expression in various mouse models of retinal degeneration. The expression construct was administered via subretinal injection packaged in AAV8BP2 . GFP fluorescence is amplified by an anti-GFP antibody and CHAT staining was performed to identify ON and OFF sublamina in the inner plexiform layer. The expression was not bipolar cell specific in any of the mouse models. The white arrows (D, H, L) indicate some of the ganglion cells expressing GFP. EGFP expression is seen in green, ChAT is seen in red and DAPI staining of the nucleus is in blue. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer

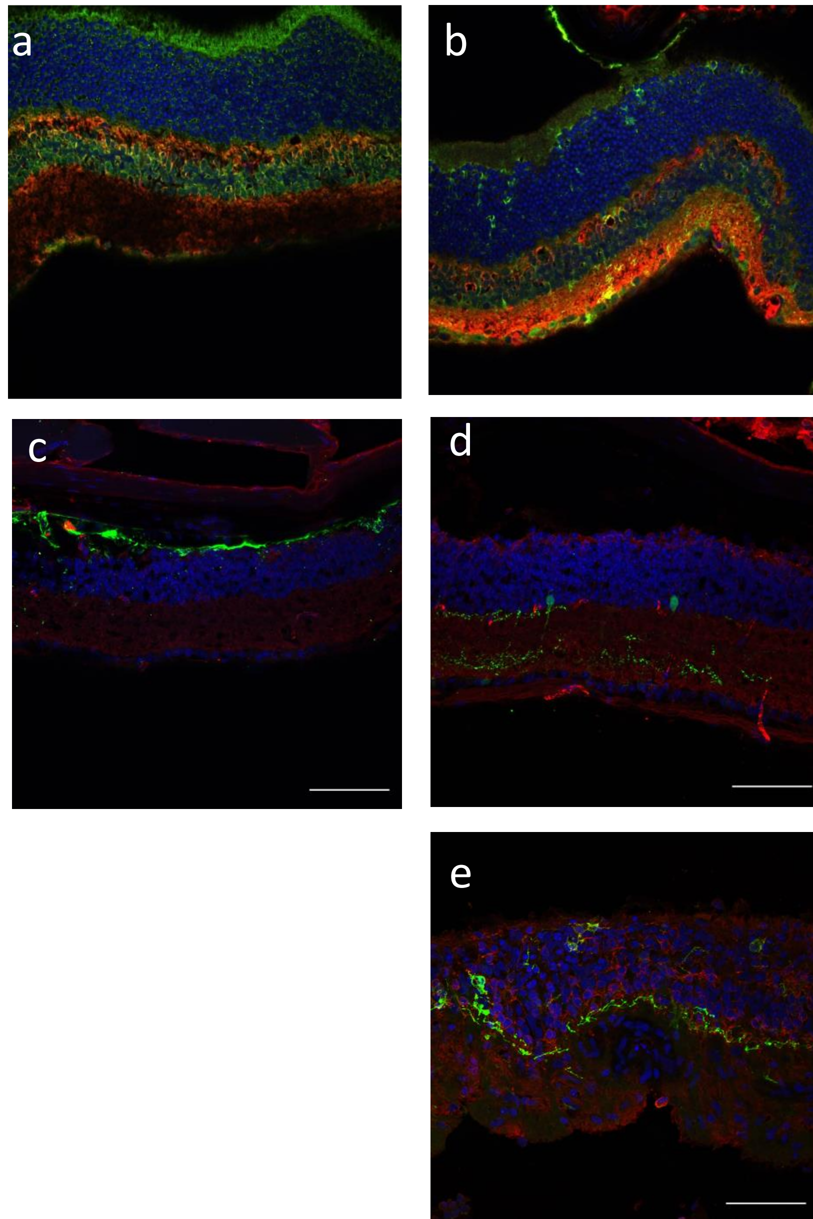

Figure 5-5: TRPM1 promoter driven expression in WT (a,b) and rd1 mice(c,d) and crd1 dog retinal explant cultures(e). AAV7m8 or AAV8BP2. eGFP expression is seen in green, GOalpha is in red and DAPI stains the nucleus blue.

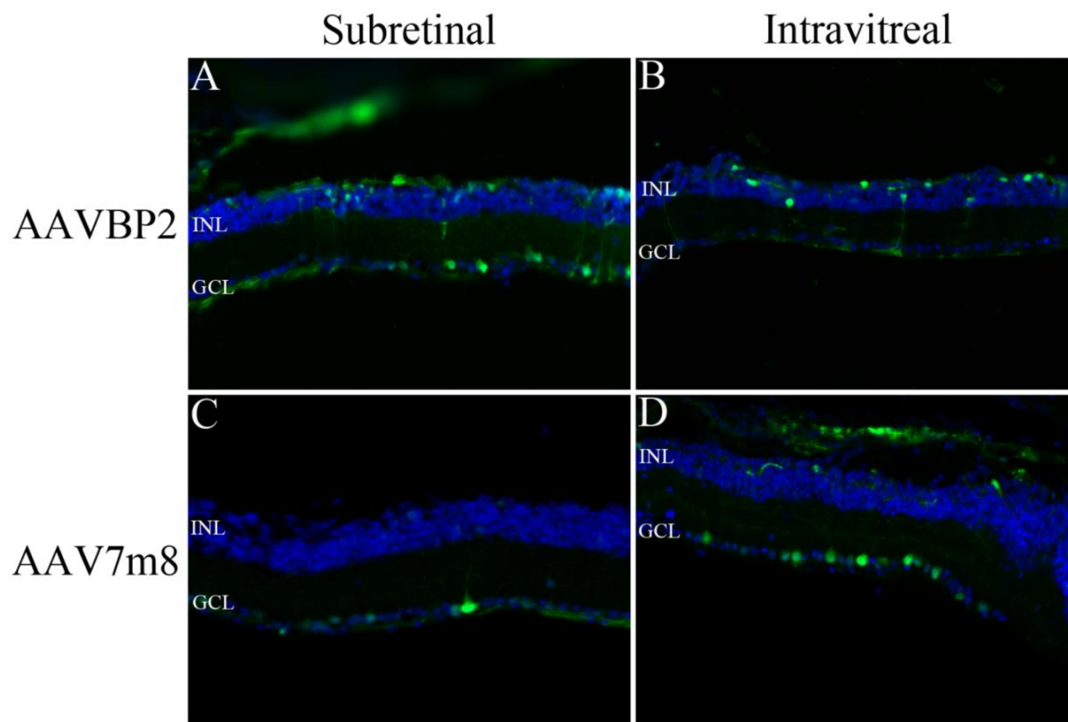

Figure 5-6: Comparison of tropism of different AAV capsids in rd1 mice. A-D: Retinal cross-section from adult rd1 mice injected with either AAV8BP2 (A and B) or AAV7m8 (C and D). GFP expression was driven by the ubiquitous CMV/CBA promoter. The virus was administered subretinally in A and C and intravitreally in B and D. ONL: outer nuclear layer, INL: inner nuclear layer, GCL: ganglion cell layer

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Chapter 6: General Conclusions and Future Directions

Filtering Whole Exome Sequencing Results: Advantages and Disadvantages

Whole exome sequencing (WES) is a powerful tool that provides a very large data set. Everyone has many rare variants in their exome, most of which are completely benign. Even *de novo* variants are usually benign, with everyone having approximately 50 *de novo* variants on average with several variants in the exome[1]. *De novo* variants are enriched in the exome considering that it makes up only 2% of the human genome, but even most variants in the exome are benign[2]. The type of mutation also serves as a poor indicator of pathogenicity. A severe mutation like a truncation and frameshift can be tolerated in some genes while a minor single nucleotide polymorphism (SNP) can be pathogenic if it is in an important position in the protein[3-5]. Conservation of the sequence across species can give an indication of the likelihood that a mutation at that position can be tolerated with highly conserved areas less likely to tolerate alteration, but it is not a guarantee that a mutation will or will not be tolerated[6]. Due to all this uncertainty, variant analysis cannot be fully automated. Applying filters can make the list of potential pathogenic variants more manageable. However, it is important to not be over-zealous in filtering the variants. Filtering brings with it the risk of removing the pathogenic variant before analysis even begins.

We found that screening for rare variants in a curated list of retinal genes already linked to retinal degeneration created a manageable list of potential variants, with usually less than 20 genes identified on each list, most of which could be eliminated quickly. We did not filter based on whether the variants were homozygous, heterozygous or compound

heterozygous. We found that if we took inheritance pattern into account after looking up the rare variants instead of before, we avoided filtering out pathogenic variants. This allowed us to identify two subjects with *de novo* dominant mutations as well as one subject diagnosed with multiplex RP who had multiple variants in several recessive genes, suggesting that the inheritance pattern in the family may not be dominant RP but rather a coalescence of several different forms of recessive RP. It also avoids the differences in interpretation that can be caused by incomplete or inaccurate family history or, in cases where one or both parents are unknown (for example, an adopted child) as well as potential problems from a second variant that is not detected by WES such as an intronic or a large copy number variant (CNV).

Screening first for variants in genes already linked to retinal degeneration shortens the analysis time for subjects with easily identified mutations. Many gene variants have been found that can cause multiple forms of retinal degeneration, depending on the exact mutation and the presence or absence of modifying alleles. For example, *PRPH2* can cause retinitis pigmentosa or macular dystrophy[7]. *BEST1* variants can cause vitelliform macular dystrophy, autosomal recessive bestrophinopathy, autosomal dominant vitreoretinochoroidopathy or retinitis pigmentosa[8]. Therefore, screening all genes associated with retinal degeneration can identify novel phenotypes for mutations in genes already known to cause retinal degeneration. Furthermore, incomplete medical or family history, examination at an early stage of disease or examination by someone other than a retinal degeneration specialist can lead to an incomplete or inaccurate diagnosis. Screening all the known retinal degeneration genes eliminates the possibility of filtering

out the pathogenic variant if the patient phenotype was mischaracterized, incomplete or continued to develop.

Testing for Digenic Causes of Retinal Degeneration

Possibly the most interesting finding was that several of the probands had multiple heterozygous mutations in retinal pathways. These mutations, while pathogenic or likely pathogenic when homozygous, are generally tolerated in isolation. However, the cumulative effect of multiple heterozygous mutations in the same pathway or protein complex has not been extensively studied. So far, few digenic or polygenic causes of retinal degeneration have been identified. This is in part due to the increased cost and complexity of studying the cumulative effect of multiple mutations in the same pathway. It is difficult to acquire enough patients with similar mutations and enough affected family members to determine whether the mutations are likely to be pathogenic when you are dealing with multiple genes instead of a single gene. It will be important to test the effect of multiple mutations in the same complex. With CRISPR becoming more widespread, we now have the tools to test the effect of heterozygous knockout in interacting proteins in cell culture and later in animal models. One subject in our cohort had a potential digenic cause with heterozygous mutations in *BBS2* and *PCM1*, which are both parts of the BBSome[9]. Cell models created using CRISPR and zebrafish models can both be used to evaluate the effect of multiple heterozygous mutations in the BBSome on cilia structure and function[10-12].

While evaluating the effect of combining specific mutations can be challenging in mice, evaluating the effect of combined null alleles is relatively simple. There are many mouse models of homozygous null alleles for various genes. In fact, both *BBS2* and *PCM1* have mouse models with null alleles[13,14]. Crossing the two mouse lines will allow us to evaluate the effect of combined heterozygous loss of function mutations in two different genes. This is an imperfect method for evaluating the mutations since a missense mutation could be acting as a dominant negative while a null mutation cannot. On the other hand, if the missense mutation creates a hypomorphic allele evaluating the effect of a null allele could actually create a more severe model and reveal an effect where the missense mutations would not have a phenotype. It is still worthwhile to study the combined effect of heterozygous null alleles to identify which genes will and will not have an additive effect from heterozygous loss of function alleles.

Induced pluripotent stem cells (IPSCs) can be used to confirm a digenic cause of disease for cilia genes like *BBS2* and *PCM1*. The IPSCs can be differentiated to RPE or neural progenitor cells and then the effect of gene augmentation on the cilia can be evaluated[15]. If the cause truly is digenic, supplementation with either of the genes should reverse the cilia deficits.

Of course, the effect of additional heterozygous mutations may be more subtle. Some variants could be acting as disease modifiers, making the phenotype more or less severe but having no effect in an unaffected individual. Modifying alleles can either affect the mutated proteins function or trafficking or they can act by affecting the expression level of the wildtype allele. Identifying modifying alleles and evaluating their role can be

complicated without a large enough cohort of patients with the same mutation or a large family with multiple affected individuals participating in the study.

Novel Gene Discovery

Trios (the parents and the proband) are needed in order to identify novel pathogenic mutations. Without trios we cannot be sure that the mutation segregates with the disease, which is step one in validating potential variants. It is possible to identify novel pathogenic variants in genes already known to cause disease without parental DNA since we already have reason to suspect that a rare variant in that gene can cause a similar phenotype to that of the patient. It can also be possible to identify novel genes if you have enough patients with the same diagnosis and a reason to believe that all or most would have the same genetic cause. For example, if the disease has stereotypical symptoms, onset and progression it is more likely to have a single genetic cause than if the disease is highly variable in onset, progression, severity and symptoms. In our case, we were studying a highly heterogeneous group and would not expect to see a high number of patients with the same novel genetic cause of their disease. Without trios we cannot reliably differentiate between a novel but benign polymorphism and a pathogenic variant without investing significant time and resources into functional testing. Proceeding to functional testing without segregation analysis would result in a lot of dead ends, with most variants in novel genes likely not having a functional effect.

In this project, we identified the patients in our cohort who are the best candidates for novel gene discovery. The next step will be to identify likely novel variants that could

lead to retinal degeneration. One method of screening for novel genes is to filter based on genes expressed in the retina. The retinome has been identified and can easily be used as a filter[24]. However, this results in a very large list of genes, including many that are not expressed in cell types likely to lead to the types of retinal degeneration we are studying. For example, mutations in bipolar cells can cause congenital stationary night blindness, but would likely not cause primary photoreceptor degeneration[25]. Instead of using such a broad set of genes to filter the results, we will be using lists of genes in the same pathway as genes that have been shown to cause retinal degeneration. Since we maintain the lists separately, we can prioritize genes based on the specific diagnosis. If we are unable to identify a likely pathogenic mutation from the pathway based analysis, we can then broaden the search to include all genes known to be expressed in the RPE and photoreceptors.

The Limits of Whole Exome Sequencing

WES has a few well known limitations. The primary drawback is that we are unable to detect intronic variants using WES. This is usually not a problem since the majority of pathogenic variants are located in the exome or splice sites (which are included in WES). Some genes, like *CEP290* and *ABCA4* have been shown to have pathogenic mutations in the introns[16,17]. Patients with LCA should always be screened for the deep intronic variant in *CEP290* before WES due to its particularly high frequency. Another type of mutation that is missed by WES are copy number variants (CNVs). With sufficient depth of coverage it is possible to see evidence of likely CNVs with WES but that is currently not a reliable method of detecting CNVs[18,19]. Despite these drawbacks, WES is still an

effective method of detecting pathogenic variants in most cases due to the rarity of CNVs and intronic variants. If a pathogenic heterozygous variant is identified, the gene should be screened for CNVs and intronic variants. If no likely pathogenic variants are identified by WES, it then makes sense to use WGS to try to find intronic mutations and CNVs.

Some diseases are not well suited for WES. Mutations in only three different genes have been shown to cause Stargardt disease, with the majority of patients having mutations in *ABCA4*[20-22]. If the initial panel screening genes associated with Stargardt/macular dystrophy comes back negative, it would be more cost and time effective to then do a complete screening of ABCA4 for intronic variants and CNVs than to perform WES. Only if those screens come back negative would it make sense to proceed to whole exome sequencing. Choroideremia is only known to be caused by mutations in *CHM*, many of which involve large deletions[23]. Evaluating choroideremia patients with WES would provide less information at a higher cost than a thorough screening of *CHM*.

Characterization of Gene Expression During Retinal Degeneration: An Interrogation using GRM6

In addition to identifying pathogenic mutations, next generation sequencing can be used to better understand the changes that occur in the retina during retinal degeneration. As we saw when we used the 4x*GRM6-SV40* promoter, retinal cells undergo profound structural and functional changes during degeneration. Although the inner retinal cells remain, the expression profile is significantly changed. Single cell RNA-Seq would

allow us to see changes in the entire transcriptome rather than evaluating gene expression on a gene by gene basis. Evaluating the transcriptome of the retina as a whole would be misleading due to the loss of the photoreceptors. A relative upregulation of proteins expressed in the inner retina is to be expected since the cell population would be enriched for those cells. Performing RNA-Seq using the entire retina would also prevent us from being able to determine if the transcripts are being expressed in different cell types than in the healthy retinal cells. Single cell analysis allows us to see the number and type of cells expressing each transcript as opposed to total transcript amount.

The expression profile of the 4x*GRM6-SV40* promoter appears to be similar to the expression profile of *GRM6* during development. While mGluR6, the protein encoded for by *GRM6*, is restricted to ON bipolar cells in healthy adult retina, it was seen in rat retinal ganglion cells both during retinal development and post-axotomy in WT mice[26,27]. It is also upregulated in response to increased retinal pressure[27]. Importantly, in our gene transfer study in the *rd1* mouse, the drastic dysregulation of the promoter does not appear to occur until there is complete loss of cone photoreceptors. In order to thoroughly evaluate the effect of retinal degeneration on the expression profile of the *GRM6-SV40* promoter, we could cross the *rd1* mouse line with the transgenic mouse line expressing GFP under the *GRM6-SV40* promoter. Using a transgenic mouse line would eliminate all confounding factors such as viral tropism, age at injection and uneven viral distribution across the retina.

Optimizing Optogenetic Therapy in Light of Retinal Remodeling

While retinal remodeling affects the expression profile of the cells in the retina, it is also likely that optogenetic therapy will have an effect on retinal remodeling. The earliest stage of retinal remodeling in rod dystrophy is for the rod bipolar cells to form synapses on the cones[28]. It is possible that if we introduce channelrhodopsin in the ON bipolar cells, it could cause ectopic synapse formation onto the ON bipolar cells, which could cause problems with retinal processing and degrading the visual signal. The best way to evaluate this would be to create a transgenic mouse line expressing channelrhodopsin under the *GRM6-SV40* promoter using the *rd1* mouse strain[29]. This would allow us to model the effect of exogenous opsin expression without including the potential variables introduced by subretinal injection and viral transduction.

AAV8b was developed through directed evolution specifically to target bipolar cells[30]. If we want to target bipolar cells in the degenerate retina, directed evolution should be done using a degenerate retina to account for the changes in receptor expression which are likely to affect viral tropism. Based on our results, optimizing viral tropism for the WT retina does not appear to translate well to the degenerate retina. Using cultured human retinal explants from donors with retinal degeneration would be the most accurate way to predict which viral capsid will target human bipolar cells effectively. Additionally, we should use a mouse model of retinal degeneration like the *rd1* or *rd10* strains since the presence of RPE and overall ocular environment may affect viral transduction efficiency. Ideally, the capsid selected should work well in both mice with retinal degeneration and cultured (and eventually *in vivo* if warranted by safety profile) human retina. As we have

shown here, using WT mice should be avoided as a model for optimizing optogenetic therapy.

Assuming that promoter expression profiles will be the same in healthy and degenerate retina has also been shown to be misleading. Instead, promoter selection and optimization should occur in the tissue type and state that will be used. Human retinal explants from donors with retinal degeneration could be used for initial promoter screening. A single retina can be cultured in multiple wells, allowing for a more efficient screen.

General Conclusion

If we want to be able to treat inherited retinal degenerations, we will need to better understand both the genetics of the disease(s) and the changes that occur in the retina during late stage retinal degeneration. Gene augmentation is still the best potential treatment option for retinal degeneration, but requires the accurate identification of the pathogenic mutations/genes. In the era of personalized medicine, we are making great strides towards the goal of identifying the pathogenic mutation in every patient, but we still need to optimize the genetic screening to minimize the amount of time needed to analyze the results and to filter the variants to a manageable list. Late stage treatment will require a thorough understanding of the retina in an advanced stage of degeneration. Optimization of both viral serotype and promoters in degenerate retina will need to be done for the best chance of success. The potential effect of optogenetics on retinal remodeling remains to be determined.

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