

**A Novel Approach for CRISPR-dCas9-Mediated
Neuroprotective Gene Therapies for Inherited Retinal
Diseases**

by

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ABSTRACT

A Novel Approach for CRISPR-dCas9-Mediated Neuroprotective Gene Therapies for Inherited Retinal Diseases

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Eye diseases are a prominent group of diseases that are difficult to treat, with a great socioeconomic impact on healthcare systems in the world. It is estimated that more than 190 million people worldwide suffer from visual impairments and 1 in 4000 people is diagnosed with retinitis pigmentosa, one of the most common inherited retinal diseases. Although inherited retinal diseases have been researched for decades, they remain mostly incurable. However, the recent approval of the gene therapy product voretigene neparvovec for the treatment of retinitis pigmentosa and Leber congenital amaurosis showed that gene therapy offers a solution to this decades-old problem. In several cases, these approaches are not sufficient and additional neuroprotective strategies are required for a complete treatment. One possible approach to protect vision is to halt or delay neuronal loss by overexpressing neuroprotective molecules. The recent discovery of CRISPR-Cas9 and its rapid adaptation in translational studies showed that CRISPR-Cas9 will change modern medicine forever. CRISPR-Cas systems were modified to produce a set of gene editing tools that were capable of gene correction and gene regulation. CRISPR-

dCas9 activation (such as dCas9-SPH) is one of these tools that is capable of transcriptional regulation of genes. With the help of these novel tools, it is now possible to overexpress and control the dosage of neuroprotective molecules in the retina. Here, we generated several multiplexed transgenic mouse lines, including $Rho^{P23H/wt}$; Tg(Rho-Cre); ROSA26-LSL-tdTomato which is a novel rod reporter mouse model with a slow retinal degeneration. This mouse model was further improved by crossing to CRISPR-dCas9-SPH activator mouse line to generate a neuroprotective test platform, the $Rho^{P23H/wt}$; Tg(Rho-Cre); ROSA26-LSL-tdTomato; dCas9-SPH-LSL quadruple transgenic mouse line. This model was complemented with AAVs which is the most commonly used gene therapy delivery tool in the ophthalmology field. AAV vectors are engineered to deliver both Cre-recombinase gene to activate the dCas9-SPH and the gRNAs to establish the artificial transcription factors in retina. Establishment of artificial transcription factors within the retina allows the overexpression of neuroprotective factors like *Lif*, *Cntf*, *Fgf2* and *Manf*. Furthermore, this model can be used as a platform to test the effects of neuroprotective factors in the degenerating retina. An additional transgenic model, $Pde6b^{rd10/rd10}$; dCas9-SPH, was also established to test the efficacy of neuroprotective factors in a fast retinal degeneration model. Further work is needed to fully characterize these transgenic models and to test the effects of different combinations and dosages of neuroprotective factors in different retinal degeneration models. This will also show whether early intervention of eye diseases with neuroprotective factors can alleviate disease progression and preserve visual function. In addition, the recently generated transgenic mouse models will be further used for several proof-of-concept studies that will open the way to generate new gene therapy products with more complex gene regulatory functions.

ÖZET

Kalıtsal Retina Hastalıkları için CRISPR-dCas9-Aracılı Nöroprotektif Gen
Tedavilerine Yeni Bir Yaklaşım

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Anahtar Kelimeler: AAV, retinitis pigmentosa, yapay transkripsiyonel faktör,
CRISPR, gen terapisi, nöroproteksiyon

Göz hastalıkları, dünyadaki sağlık sistemleri üzerinde büyük bir sosyoekonomik etkiye sahip, tedavisi zor bir hastalık grubudur. Dünya çapında 190 milyondan fazla insanın görme bozukluğu yaşadığı ve 4000 kişiden 1'inde en yaygın kalıtsal retina hastalıklarından biri olan retinitis pigmentosa teşhisi konduğu tahmin edilmektedir. Kalıtsal retina hastalıkları onlarca yıldır araştırılsa da, çoğunlukla tedavi edilemez durumdadırlar. Bununla birlikte, retinitis pigmentosa ve Leber konjenital amarozişi tedavisi için gen tedavisi ürünü voretigene neparvovec'in yakın zamanda onaylanması, gen tedavisinin on yıllardır süren bu soruna bir çözüm sunabileceğini gösterdi. Bazı durumlarda tam bir tedavi için ek nöroprotektif stratejiler gereklidir. Nöronal kaybı durdurmak veya geciktirmek, nöroprotektif molekülleri aşırı ifade etmek, görmeyi korumaya yönelik olası yaklaşımlardır. CRISPR-Cas9'un yakın zamanda keşfi ve translasyon çalışmalarına hızlı adaptasyonu, CRISPR-Cas9'un

modern tıbbı sonsuza dek deęiřtireceęini gsterdi. CRISPR-Cas sistemleri, gen dzeltme ve gen dzenleme yeteneęine sahip bir dizi gen dzenleme aracı retecek řekilde modifiye edildi. CRISPR-dCas9 aktivasyonu (dCas9-SPH gibi), genlerin transkripsiyonel reglasyonu iin kullanılan tekniklerden biridir. Bu yeni araların yardımıyla, retinadaki nroprotektif moleklleri ařırı ifade etmek ve dozunu kontrol etmek artık mmkn. Bu alıřmada, yavař retinal dejenerasyona sahip yeni bir ubuk raportr fare modeli olan $Rho^{P23H/wt}$; Tg(Rho-Cre); ROSA26-LSL-tdTomato dahil olmak zere birka oklanmış transgenik fare modeli retilmiřtir. Bu fare modeli, nroprotektif bir test platformu oluřturmak iin CRISPR-dCas9-SPH aktivatr fare hattı ile iftleřtirilerek daha da geliřmiř bir drtl transgenik fare hattı olan $Rho^{P23H/wt}$; Tg(Rho-Cre) ; ROSA26-LSL-tdTomato ; dCas9-SPH-LSL retildi. Bu model, oftalmoloji alanında en sık kullanılan gen tedavisi uygulama aracı olan AAV'ler ile tamamlanmıřtır. AAV vektrleri, dCas9-SPH'yi aktive etmek iin hem Cre-rekombinaz genini hem de retinada yapay transkripsiyon faktrlerini oluřturmak iin gRNA'ları teslim etmek zere tasarlanmıřtır. Retina iinde yapay transkripsiyon faktrlerinin oluřturulması, *Lif*, *Cntf*, *Fgf2* ve *Manf* gibi nroprotektif faktrlerin ařırı ekspresyonunu mmkn kılmıřtır. Ayrıca, bu model, dejenere olan retinadaki nroprotektif faktrlerin etkilerini test etmek iin bir platform olarak da kullanılabilir. Hızlı bir retinal dejenerasyon modelinde nroprotektif faktrlerin etkinlięini test etmek iin de ek bir transgenik model, $Pde6b^{rd10/rd10}$; dCas9-SPH, kurulmuřtur. Bu transgenik modelleri tam olarak karakterize etmek ve farklı retinal dejenerasyon modellerinde nroprotektif faktrlerin farklı kombinasyon ve dozajlarının etkilerini test etmek iin daha fazla alıřmaya ihtiya vardır. Bu aynı zamanda nroprotektif faktrlerle gz hastalıklarına erken mdahalenin hastalıęın ilerlemesini hafifletip, grme fonksiyonunu koruyup koruyamayacaęını da gsterecektir. Ek olarak, yakın zamanda retilen transgenik fare modelleri, daha karmařık gen dzenleyici iřlevlere sahip yeni gen tedavisi rnleri retmenin yolunu aacak olan birka kavram kanıtı alıřmasında da kullanılacaktır.

TABLE OF CONTENTS

1. INTRODUCTION.....	1
1.1. Purpose.....	1
1.2. The Human Eye and The Retina.....	2
1.3. Visual Cycle.....	7
1.4. Inherited Retinal Diseases.....	9
1.4.1. Non-Syndromic Retinitis Pigmentosa (RP)	9
1.4.2. Mouse Models of IRDs.....	11
1.4.2.1. P23H Mouse Line.....	11
1.4.2.2. Rd10 Mouse Line.....	12
1.5. Gene Therapy and Gene Editing Tools.....	13
1.5.1. Adeno-Associated Virus (AAV).....	15
1.5.2. Gene Editing and Regulatory Tools.....	16
1.6. Neuroprotection in Retina.....	22
2. MATERIALS AND METHODS.....	25
2.1. Animal Breeding Strategy.....	25
2.1.1. Rod Reporter and P23H Rod Reporter Mouse Lines.....	25
2.1.2. P23H-SPH Mouse Line.....	27
2.1.3. Rd10-SPH Mouse Line.....	28
2.2. Genotyping.....	28
2.2.1. Toe Biopsy.....	28
2.2.2. Polymerase Chain Reaction (PCR).....	29
2.2.2.1. P23H PCR.....	32
2.2.2.2. Ai14 Wt and Mutant PCR.....	32
2.2.2.3. Rd10 PCR.....	33
2.2.2.4. dCas-SPH PCR.....	34
2.2.3. Gel Electrophoresis.....	35
2.2.4. Fluorescent and Bright-Field Fundus Imaging.....	36

2.3. Optical Coherence Tomography (OCT).....	37
2.4. Cloning.....	38
2.4.1. sgRNA Amplification.....	38
2.4.2. PCR Purification.....	38
2.4.3. Restriction Digestion.....	39
2.4.4. Gel Extraction.....	40
2.4.5. Ligation.....	40
2.4.6. Competent Cell Preparation.....	41
2.4.7. Transformation.....	42
2.4.8. Colony PCR.....	42
2.4.9. Small-Scale Plasmid Isolation.....	43
2.4.10. ITR Stability Check.....	43
2.4.11. Sanger Sequencing.....	44
2.4.12. Large-Scale Plasmid Isolation.....	44
2.5. Large-Scale AAV Production.....	45
2.5.1. Cell Culture.....	45
2.5.1.1. Cell Thawing.....	45
2.5.1.2. Cell Passaging.....	46
2.5.1.3. Cell Freezing.....	46
2.5.2. Transfection.....	48
2.5.2.1. Polyethylenimine (PEI) Preparation.....	48
2.5.2.2. PEI Transfection.....	48
2.5.3. AAV Harvest.....	48
2.5.3.1. Poly(ethylene glycol) (PEG) Preparation.....	48
2.5.3.2. From Supernatant.....	49
2.5.3.3. From Cells.....	49
2.5.4. Iodixanol Solution Preparation.....	50
2.5.5. Iodixanol Gradient Preparation.....	50
2.5.6. AAV Fraction Purification.....	51
2.5.7. DNase Treatment.....	51
2.5.8. Digital Droplet PCR (ddPCR) Titration.....	51
2.6. Intravitreal Injection.....	53
2.7. Fluorescent Activated Cell Sorting.....	54
2.7.1. Whole Retina Isolation and Dissociation.....	55

2.7.2. Cell Sorting.....	56
2.8. Whole Retina Snap-Freeze.....	56
2.9. cDNA Library.....	56
2.9.1. From Sorted Cells.....	57
2.9.2. From Whole Retina.....	57
2.9.2.1. RNA Extraction.....	57
2.9.2.2. cDNA Production.....	58
2.10. ddPCR.....	59
2.11. Statistical Analysis.....	59
 3. RESULTS.....	 60
3.1. Generation of a P23H Rod Reporter Mouse Model.....	60
3.1.1. P23H Rod Reporter Mouse Line Follow-up.....	62
3.2. pAAV-sgRNA Vector Cloning.....	62
3.2.1. pAAV-sgRNA Vector Design.....	63
3.2.2. Confirmation of dCas9-SPH-Mediated Overexpression <i>in vitro</i>	65
3.3. AAV-sgRNA Titration.....	66
3.4. AAV-sgRNA Cre Recombinase Activity.....	67
3.5. dCas-SPH Mice Characterization.....	69
3.6. <i>In vivo</i> Activity of dCas-SPH.....	70
3.7. Rd10-SPH Follow-up.....	73
3.8. dCas-SPH Activity in a Fast Degeneration Mouse Model.....	74
3.9. dCas-SPH Activity in a Slow Degeneration Mouse Model.....	77
 4. DISCUSSION.....	 82
 5. BIBLIOGRAPHY.....	 87
 6. APPENDIX.....	 94

LIST OF FIGURES

- Figure 1. The simplified anatomy of the eye.
- Figure 2. The simplified anatomy and neuronal network of the retina.
- Figure 3. A diagram of the canonical visual cycle and relevant IRDs associated with key enzymes in the visual cycle.
- Figure 4. Commonly used gene replacement and gene correction tools.
- Figure 5. Gene regulatory tools for altering the transcriptional profile of genes.
- Figure 6. IL-6 cytokine family receptors and their different combinations.
- Figure 7. Breeding strategy for the rod reporter and P23H rod reporter mouse models.
- Figure 8. Breeding strategy for the P23H-SPH mouse model.
- Figure 9. Breeding strategy for the rd10-SPH mouse model.
- Figure 10. P23H genotyping visualized by gel electrophoresis.
- Figure 11. Ai14 genotyping PCR visualized by gel electrophoresis.
- Figure 12. Rd10 genotyping PCR visualized by gel electrophoresis.
- Figure 13. dCas-SPH genotyping PCR visualized by gel electrophoresis.
- Figure 14. Ai14 leakage and Ai14-Tg(Rho-Cre) rod reporter activity visualization through retinal imaging.
- Figure 15. ITR stability testing of pAAV-sgRNA vectors.
- Figure 16. Large-scale AAV production.
- Figure 17. Intravitreal AAV-sgRNA injection readout.
- Figure 18. P23H rod reporter mouse model follow-up study.
- Figure 19. A map of the PCA01 plasmid.
- Figure 20. A map of pAAV-Cre plasmid.
- Figure 21. Cloning strategy for pAAV-sgRNA.
- Figure 22. Testing the *in vivo* Cre recombinase activity of AAV-sgRNA.

Figure 23. Fundus features of dCas-SPH mice.

Figure 24. AAV-sgRNA-mediated *in vivo* dCas-SPH activation.

Figure 25. AAV-sgRNA *in vivo* safety test.

Figure 26. Characterization of Rd10-SPH mice retina.

Figure 27. Viral load testing in Rd10-SPH retinas.

Figure 28. Single and combined AAV-sgRNA *in vivo* trial.

Figure 29. Fluorescent fundus imaging of *Fgf2*, *Manf*, *Lif*, *Cntf* overexpression in a slow degeneration mouse model.

Figure 30. OCT imaging of *Fgf2*, *Manf*, *Lif*, *Cntf* overexpression in a slow degeneration mouse model.

Figure 31. Retinal thickness and rhodopsin expression levels of AAV-sgRNA treated retinas.

LIST OF TABLES

Table 1. Tissue lysis buffer.

Table 2. Neutralization buffer.

Table 3. GoTaq Green master mix.

Table 4. Standard PCR reaction.

Table 5. Ai14 PCR protocol.

Table 6. dCas-SPH PCR protocol.

Table 7. Rd10 PCR protocol.

Table 8. High-fidelity PCR reaction.

Table 9. High-fidelity PCR protocol.

Table 10. Restriction digestion.

Table 11. Ligation reaction.

Table 12. Colony PCR.

Table 13. Standard ddPCR Reaction.

Table 14. ddPCR Protocol.

Table 15. Reverse transcription reaction for sorted cells.

Table 16. Reverse transcription reaction for whole retina.

Table 17. Reverse transcription protocol.

Table 18. Titers of AAV-sgRNA.

Table 19. Cloning primers.

Table 20. Genotyping primers.

LIST OF ABBREVIATIONS

AAV: Adeno-Associated Virus
ABCA4: ABC Transporter
ABE: Adenine Base Editor
Ad: Adenovirus
Adrp: Autosomal Dominant Retinitis Pigmentosa
AMD: Age-Related Macular Degeneration
ASO: Antisense Oligonucleotide
Arrp: Autosomal Recessive Retinitis Pigmentosa
ATF: Artificial Transcription Factor
BDNF: Brain-Derived Neurotrophic Factor
BM: Bruch's Membrane
BP: Base Pair
Cas: CRISPR-Associated Protein
Cas9n: Cas9-Nickase
CBA: Chicken Beta Actin Promoter
CBE: Cytidine Base Editor
cDNA: Complementary DNA
cGMP: Cyclic Guanosine Monophosphate
CNG: Cyclic Nucleotide-Gated
CNTF: Ciliary Neurotrophic Factor
COS: Cone Outer Segments
CRALBP: Cellular Retinaldehyde Binding Protein
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
crRNA: CRISPR RNA
dCas: Nuclease Dead Cas
ddH₂O: Double Distilled Water
ddPCR: Digital Droplet Polymerase Chain Reaction
DMEM: Dulbecco's Modified Eagle's Medium
DNA: Deoxyribonucleic Acid
dNTP: Deoxynucleoside triphosphate
DSB: Double-Stranded Break

EDTA: Ethylenediaminetetraacetic Acid
EGFP: Enhanced Green Fluorescent Protein
ELM: External Limiting Membrane
EMA: European Medicines Agency
ERG: Electroretinogram
ERK: Extracellular Signal-Regulated Kinase
FACS: Fluorescence-Activated Cell Sorting
FAZ: Foveal Avascular Zone
FBS: Fetal Bovine Serum
FDA: Food and Drug Administration
FGF2: Basic Fibroblast Growth Factor
FITC: Fluorescein Isothiocyanate
FSC: Forward Scatter
GC: Guanylyl Cyclase
GCL: Ganglion Cell Layer
GFP: Green Fluorescence Protein
GFP: Green Fluorescent Protein
GPCR: G-Protein-Coupled Receptor
gRNA: Guide RNA
gRNA: Guide RNA
HDR: Homology-Directed Repair
HEK293T: Human Embryonic Kidney 293T
HSF: Heat-Shock Factor
IGF: insulin-like growth factor
IL-6: Interleukin-6
ILM: Inner Limiting Membrane
INL: Inner Nuclear Layer
IPL: Inner Plexiform Layer
IRBP: Interphotoreceptor Retinoid-Binding Protein
IRD: Inherited Retinal Disorders
IRS-1: Insulin Receptor Substrate 1
ITR: Inverted Terminal Repeat

JAK: Janus Kinase
kb: Kilobase
LB: Lysogeny Broth
LCA: Leber Congenital Amaurosis
LCA2: LCA Type 2
LGN: Lateral Geniculate Nucleus
LIF: Leukemia Inhibitory Factor
LRAT: Lecithin Retinol Acyltransferase
MANF: Mesencephalic Astrocyte-Derived Neurotrophic Factor
MAPK: Mitogen-Activated Protein Kinase
MgCl₂: Magnesium Chloride
M-MLV: Moloney Murine Leukaemia Virus
MSVI: Medium-to-Severe Visual Impairment
NaCl: Sodium Chloride
NFL: Nerve Fiber Layer
NHEJ: Non-Homologous End Joining
OCT: Optical Coherence Tomography
ONL: Outer Nuclear Layer
OPL: Outer Plexiform Layer
Opn1mw: Medium Wavelength Opsin
OS: Outer Segments
P23H: Pro23His Mutation
PAM: Protospacer Adjacent Motif
PBS: Phosphate-Buffered Saline
PCR: Polymerase Chain Reaction
PDE: Phosphodiesterase
PDE6B: Phosphodiesterase 6B
PEG: Poly(ethylene glycol)
pegRNA: Prime Editing gRNA
PEI: Polyethyleneimine
PEI: Polyethylenimine
Pen-Strep: PS, Penicillin-Streptomycin

PL: Photoreceptor Layer
PND: Postnatal Day
PNW: Post Natal Week
rAAV: Recombinant AAV
RAL: Retinal
RDH: Retinol Dehydrogenase
RE: Retinyl Ester
RGC: Retinal Ganglion Cell
RHO: Rhodopsin
RNA: Ribonucleic Acid
ROL: Retinol
ROS: Rod Outer Segments
RP: Retinitis Pigmentosa
RPE: Retinal Pigment Epithelium
RPE65: Retinal Pigment Epithelium 65
RT: Reverse Transcriptase
RVD: Repeat Variable Diresidue
scFv: Single Chain Variable Fragment
sgRNA: Single Guide RNA
SPH: SunTag-p65-HSF1
sRP: Simplex Retinitis Pigmentosa
SSC: Side Scatter
STAT: Signal Transducer and Activator of Transcription Proteins
TAE: Tris Acetic Acid EDTA
TALE: Transcriptional Activator-Like Effector
TALEN: Transcriptional Activator-Like Effector Nuclease
tdTomato: Tandem Dimer Tomato
tracrRNA: *trans*-Activating CRISPR RNA
UGI: Uracil Glycosylase Inhibitor
UPR: Unfolded Protein Response
VEGF: Vascular Endothelial Growth Factor
VN: Voretigene Neparvovec, Luxturna

VPR: VP64, p65, Rta Fusion Protein

XLN: X-Linked Recessive

ZF: Zinc Finger

ZFN: Zinc Finger Nuclease

ZFP: Zinc Finger Protein



1. INTRODUCTION

1.1. Purpose

Eyes are arguably one of the most important organs in the human body as they provide the sense of vision, and assistance in communication and navigation. As with most organs in the human body, the functionality of the eye deteriorates, and it is more prone to diseases with the aging of a person. It is evident that European countries are experiencing aging of their populations due to an increase in the life expectancy of people [1]. Thus, eye diseases are one of the top socioeconomic burdens on healthcare systems worldwide as well as European countries. It is estimated that 32,4 million people are blind while 191 million people suffer from medium-to-severe visual impairment (MSVI) worldwide [2]. The estimated socioeconomic burden of visually impaired people are €25,8 billion in Europe alone [3]. Age-related macular degeneration (AMD) is a multifactorial macular disease that is the main cause of blindness in people over 55 years old [4]. It is estimated that AMD will affect 19 million people in Europe by the year 2040 [5]. Older age is the strongest risk factor for AMD, and smoking is consistently listed as a risk factor as well [6, 7]. Mutations in genes related to complement activation pathways and immune processes have been associated with AMD. However, the heritability of AMD is still being researched with only half of the genomic heritability explained in the literature. There are various classifications for AMD such as early, late, neovascular, and atrophic AMD. Early AMD usually goes unnoticed due to slow progression and absence of symptoms. Late AMD can progress rapidly in several weeks to months due to neovascularization or progression can take years in atrophic AMD [8].

Inherited retinal diseases (IRDs) are a group of diseases that is a major cause of blindness in the world. IRDs are genetically heterogeneous and they can cause retinal degeneration due to a loss of retinal ganglion cells, pigment epithelium and photoreceptor cells. The progression of retinal degeneration, age of onset, and the prognosis of an IRD is affected by genetic and environmental factors, and thus they can vary greatly. The most common subgroups of IRDs are retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA) [9]. Diseases of the retina and macula are still untreated. The common strategy is the treatment of symptoms and increasing the quality of life of the patients. Recently, gene editing tools that offer a wide variety of treatment options have emerged. Gene therapy is a medical approach that is aimed to prevent or cure a disease by modifying the DNA or RNA of an organism. Voretigene neparvovec (VN, Luxturna) became the first successful gene therapy product that corrects the underlying mutation causing retinitis pigmentosa and Leber congenital amaurosis [10, 11]. Following the success of VN, copious amounts of gene therapy clinical trials have started and are still underway. We aimed to produce a novel rod cell reporter retinal degeneration mouse model to better characterize retinal atrophy and rod cell loss. Furthermore, a platform of clustered regularly interspaced short palindromic repeats (CRISPR)-dCas9-mediated transcriptional regulation of neuroprotective factors in multiple retinal degeneration mouse models was generated. This thesis is aimed to produce a platform to enable the production and testing of novel gene therapy products for the treatment of eye diseases.

1.2. The Human Eye and The Retina

The eye is a sensory organ that is capable of receiving light under various complex situations and transforming this information into signals to be sent to the brain. Eyes are a part of the central nervous system, however, eyes are mostly isolated from the rest of the body. The eye is in a distorted sphere shape that is made of three layers called external, intermediate and internal layer. The external layer is made of cornea on the superior portion of the eye while the rest is made of sclera. Cornea covers the pupil and the iris, and functions as the main focusing lens of the eye. Sclera is the white of the eye that provides structure and protection to the eye. Intermediate layer is made of the iris and

ciliary body on the anterior and the choroid on the posterior. Iris is located under the cornea, and the anterior chamber filled with aqueous humor is located between the iris and cornea. Under the iris is the posterior chamber filled with aqueous humor that connects the lens to the iris. Crystalline lens is the second focusing lens of the eye. It is suspended in place by zonule fibres connected to the ciliary muscles. Contrary to the

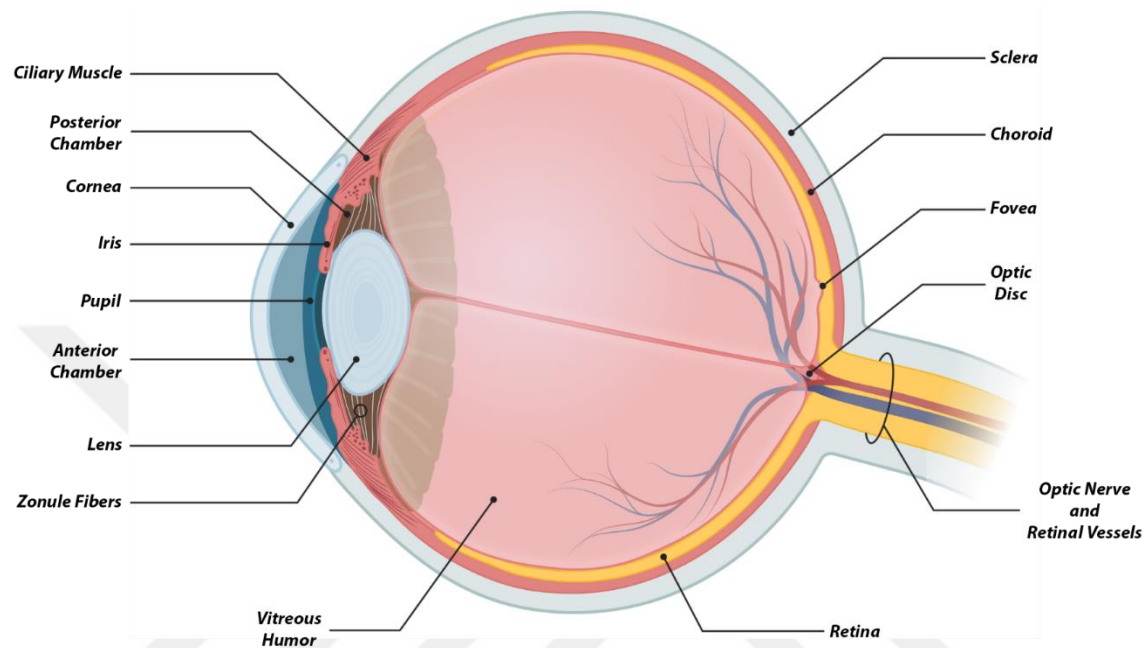


Figure 1. The simplified anatomy of the eye. The eye contains cornea and the sclera at the external layer, iris, ciliary body and the choroid at the intermediate layer, and retina at the inner layer. Cornea is a stationary lens while the crystalline lens can change its shape with the help of ciliary muscles and zonule fibers. Anterior and posterior chambers are filled with aqueous humor and they fill the space between cornea and iris, and iris and lens, respectively. Iris and pupil are covered by the cornea. Iris blocks the light from coming in, restricting light to access retina through pupil. The interior of the eye is filled by vitreous humor. Retina is the light-sensing neuronal part of the eye. Retina contains the cone exclusive region of the retina called fovea and the cone rich region surrounding the fovea, called macula. Retinal ganglion cells (RGCs) transport the light signal to the brain through the optic nerve. The area around the optic nerve is the optic disc. Figure adapted from “Anatomy of the Human Eye”, by BioRender.com (2022). Retrieved from <https://app.biorender.com/biorender-templates>.

cornea, lens can change shape to accommodate focus through the contraction and relaxation of the ciliary muscles. Zonule fibres suspend the crystalline lens to the choroid that is mostly made of blood vessels [12]. The essential function of the choroid is blood supply to the outer retina. It also contains other secondary functions such as thermoregulation and secretion of growth factors [13]. Finally, the neuronal sensory part of the retina resides at the innermost layer. Vitreous humor, a viscous liquid, fills the space between lens and the retina [12]. The center that mediates high-resolution vision is

called fovea and it is located at the center of the retina. The small region around fovea that is dense in cone photoreceptors is called macula. The region containing the optic nerve that is surrounded by blood vessels is called optic disc and it is nasal to the fovea [14]. Fovea and macula regions are exclusively found in some of the primates and birds. Thus, the commonly used experimental animal *mus musculus* lacks a fovea and macula [15, 16]. Fovea is a depression at the retina that is made solely of cone photoreceptors and some Müller cells. Fovea contains no retinal capillaries, forming the foveal avascular zone (FAZ) [17].

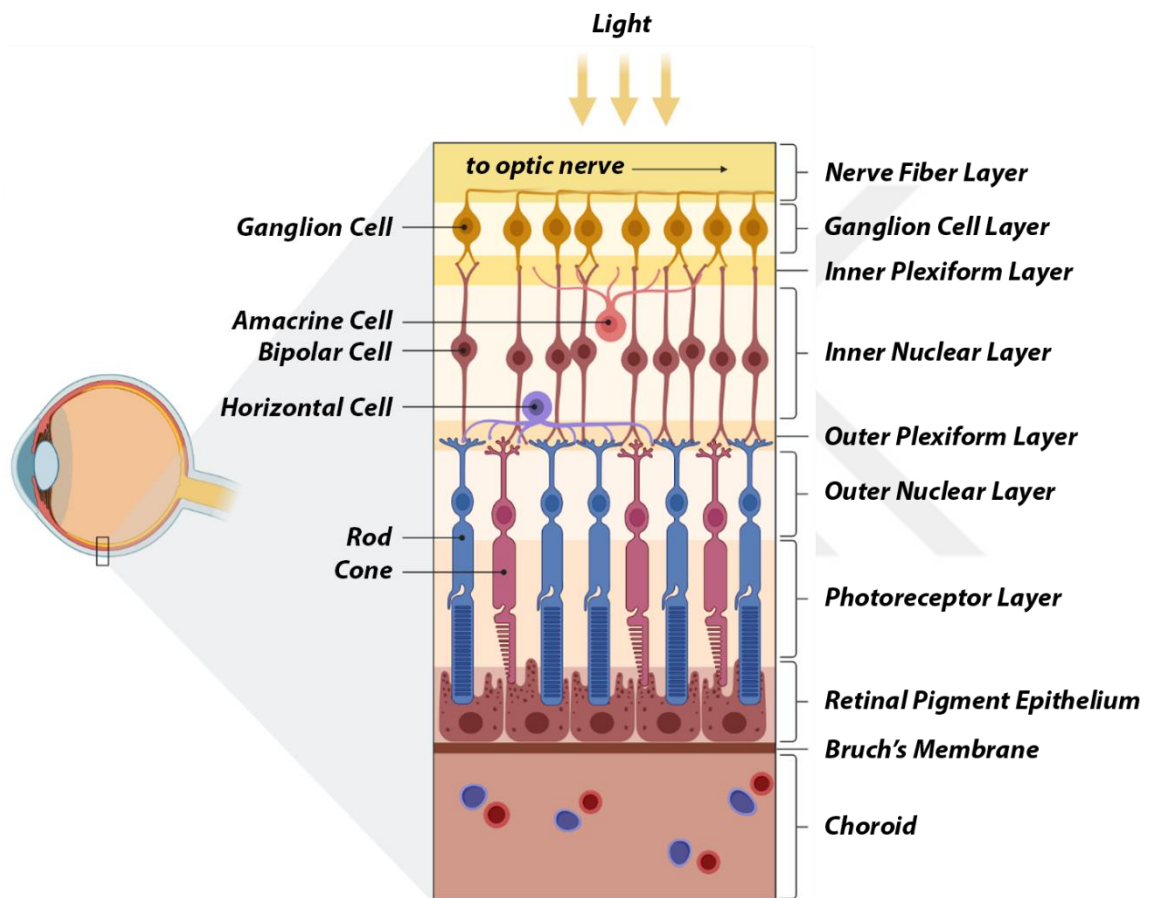


Figure 2. The simplified anatomy and neuronal network of the retina. Light hits the retina and travels through the inner layers to the photoreceptor layer where it is absorbed by rod and cone opsin proteins. The innermost layer, nerve fiber layer, contains axons of RGCs that are travelling towards the optic nerve. RGC, amacrine and bipolar cells make connections in the IPL. The bodies of amacrine, bipolar, horizontal and Müller cells are located in the INL. Photoreceptor cell axons make synapses with INL neurons in the OPL. The cell bodies of photoreceptors reside in the ONL. The photoreceptor layer contains the inner and outer segments of photoreceptor cells. RPE layer contains RPE cells that supports photoreceptor cells. At the outermost, Bruch's membrane separates the retina from the choroid. Figure adapted from "Structure of the Retina", by BioRender.com (2022). Retrieved from <https://app.biorender.com/biorender-templates>.

The retina is populated by many different cell populations that have distinct functions throughout the phototransduction and photosignaling processes. Out of 80-120 million

photoreceptors in the human retina, approximately 95% are rod while the remaining 5% are cone photoreceptors [17]. Similarly, the mouse retina contains 97% rod photoreceptors [18, 19]. Most of the cone photoreceptors in the human retina are concentrated in the fovea and macula, while the remainder of the peripheral retina contains mostly rod photoreceptors [20]. Rod cells are responsible for low light (scotopic), black-and-white vision. Rod cells are photobleached during daylight, meaning they can only function in the dark. All rod cells have the same opsin called rhodopsin. Rod cells have very low spatial acuity due to the synapses they make with the neurons in the plexiform layers. Rod bipolar cells may interact with as much as 70 rod cells while cone bipolar cells can form 1-to-1 connections. This reduces the resolution of the image, however, enables low light vision with reduced noise [20, 21]. Cone cells are less sensitive to photons, and they are more selective towards the wavelengths of photons. Almost all cone cells contain one and only one cone opsin [21]. Most mammalian retina contain two types of cone cells. One with a long wavelength opsin (red) and another with a short wavelength opsin (blue). Humans on the other hand have a third cone cell type with a medium wavelength opsin (green). Opsin types are distributed in a 16:8:1 ratio between red, green and blue opsin containing cone cells [17, 20]. Cone cells are responsible for day vision (photopic) and they adapt to changes in light much better than rod cells. Cone cells can recover from photobleaching in 20 milliseconds while rod cells can take as much as 20 minutes [20]. Rod and cone cells make synapses with either hyperpolarized (OFF) or depolarized (ON) bipolar cells. Bipolar cells carry this signal over to ganglion cells, connecting the photoreceptor cell to the RGC [17]. Amacrine cells are very complex cells that make connections to bipolar cells, ganglion cells as well as each other. They are centralized axonless cells with various roles. For example, a small population of wide-field amacrine cells can cover the whole retina in a network. Their main functions are object motion detection in contrast to self, and carrying signals through retinal layers. Horizontal cells provide an inhibitory signal to the photoreceptors, thus, they modify the gain of the image by measuring the average light intensity falling on the retina through their lateral spread processes. This basically enables the organism to perceive details on bright objects [21]. Ganglion cells are the last step that carries the photosignal to the brain. They make connections to the lateral geniculate nucleus (LGN) region of the brain. There are many different types of RGCs with large receptive fields (parasol), and some small population even photosensitive. These melanopsin containing photosensitive RGCs play a role in circadian rhythm and melatonin secretion [17, 20].

The retina is a complex structure that contains multiple layers with different types of neurons. This amazing structure processes and refines the initial light signal by passing it through the many different neural layers to output the image we see. Retina is separated from the choroid by the Bruch's membrane (BM). This membrane is made of elastin, collagen and other adhesive glycoproteins to keep the cells attached to it. Retinal pigment epithelium (RPE) is placed between the photoreceptor layer and the BM. RPE cells form this singular layer by forming tight junctions between each other, isolating the photoreceptor layer from the Bruch's membrane. RPE layer and Bruch's membrane are the main components of the blood-retina barrier that regulates the retinal function and blood supply. The photoreceptor layer resides on top of the RPE layer and it consists of the rod and cone photoreceptor inner and outer segments. This layer is the sole one responsible for light signal detection and phototransduction [17]. Rod cell outer segments (ROS) are covered in plasma membrane while cone cell outer segments (COS) are exposed to the extracellular matrix. This property most likely enhances the phototransduction and metabolism of cone cells [22]. External limiting membrane (ELM) covers the photoreceptor layer by the junctional complexes made between photoreceptor and Müller cells. The photoreceptor nuclei are located in the outer nuclear layer (ONL). Outer plexiform layer (OPL) is crucial for the photosignal processing. Photoreceptor axons make connections with the bipolar and horizontal cells of the inner nuclear layer here. Inner nuclear layer (INL) contains the nuclei of bipolar, horizontal, amacrine, interplexiform, and Müller cells stacked on top of each other in three sublayers. Inner plexiform layer (IPL) is the second center where crucial signal processing happens. Bipolar, amacrine and ganglion cells form complex connections in six lamina of the IPL. The ganglion cell layer is made of mostly retinal ganglion cells (RGCs) and displaced amacrine cells. RGC axons are located at the nerve fiber layer (NFL) and they are aimed towards the optic disc where the optic nerve head is located. The innermost retinal layer is called inner limiting membrane (ILM). ILM is made of Müller cell processes that flatten and combine with collagen to isolate the retina from the vitreous humour [17]. This complex neuronal network carries out many different functions in the retina, and most importantly, they maintain the visualization of the outer world by the host organism. The visual information starts as a photon hitting the photoreceptor outer segments and it is transformed into electrical signals through a sophisticated pathway that requires the coordination and support of many different neurons in the retina.

1.3. Visual Cycle

The key molecule in capturing photons is the visual pigment. Visual pigment consists of a light-sensing chromophore, 11-*cis*-retinal (RAL), that is covalently attached to an opsin protein. This chromophore originates from all-*trans*-retinol (all-*trans*-ROL, vitamin A) that is carried to RPE cells through the choroid. All-*trans*-ROL is esterified in the RPE cells by lecithin retinol acyltransferase (LRAT) to all-*trans*-retinyl ester (all-*trans*-RE) [22, 23]. This esterification reaction is crucial for photoreceptor and RPE cell survival as retinal is very toxic [24]. Retinal pigment epithelium 65 (RPE65) hydrolyzes and isomerizes all-*trans*-RE to 11-*cis*-ROL in a single, rate-limiting reaction. 11-*cis*-ROL, bound to a protein called cellular retinaldehyde binding protein (CRALBP), is oxidized by 11-*cis*-retinol dehydrogenase (RDH5) into 11-*cis*-RAL. 11-*cis*-RAL is transferred to outer segments of photoreceptors by interphotoreceptor retinoid-binding protein (IRBP). Thus, the regeneration of 11-*cis*-RAL from vitamin A is completed with the essential help of RPE cells [22-24].

The regenerated 11-*cis*-RAL then forms a Schiff base bond with an opsin (rhodopsin or cone opsin) protein to form the visual pigment. Opsin is a protein belonging to the G-protein-coupled receptors family and it has seven transmembrane helices. The free opsin protein (apo-opsin) has reduced activity. 11-*cis*-RAL bound to opsin acts as an antagonist in the darkness and inhibits its functions. 11-*cis*-RAL goes through a conformational change after it absorbs a photon, and becomes an opsin agonist called all-*trans*-RAL. This conformational change results in the active state of the visual pigment. The visual pigment activates the G-protein transducin and decays into opsin and all-*trans*-RAL. All-*trans*-RAL is transported to the cytosolic side of the disc membranes by ABC transporter ABCR (ABCA4). All-*trans*-RAL is then reduced to all-*trans*-ROL by NADPH-dependent all-*trans*-retinol dehydrogenase (RDH). IRBP transports all-*trans*-ROL back to the RPE to repeat the cycle and regenerate 11-*cis*-RAL [22-24]. This canonical cycle applies to both cone and rod photoreceptor cells. However, it is highly likely that cone photoreceptors are supported by an alternative cycle to replenish the visual pigment faster than rod photoreceptors. Three enzymes have been found to be performing alternatively to the

canonical light cycle in cone cells. Isomerase II catalyzes all-*trans*-ROL to 11-*cis*-ROL, retinyl ester synthase catalyzes all-*trans*-ROL to all-*trans*-RE, and RDH catalyzes 11-*cis*-ROL to 11-*cis*-RAL. [22].

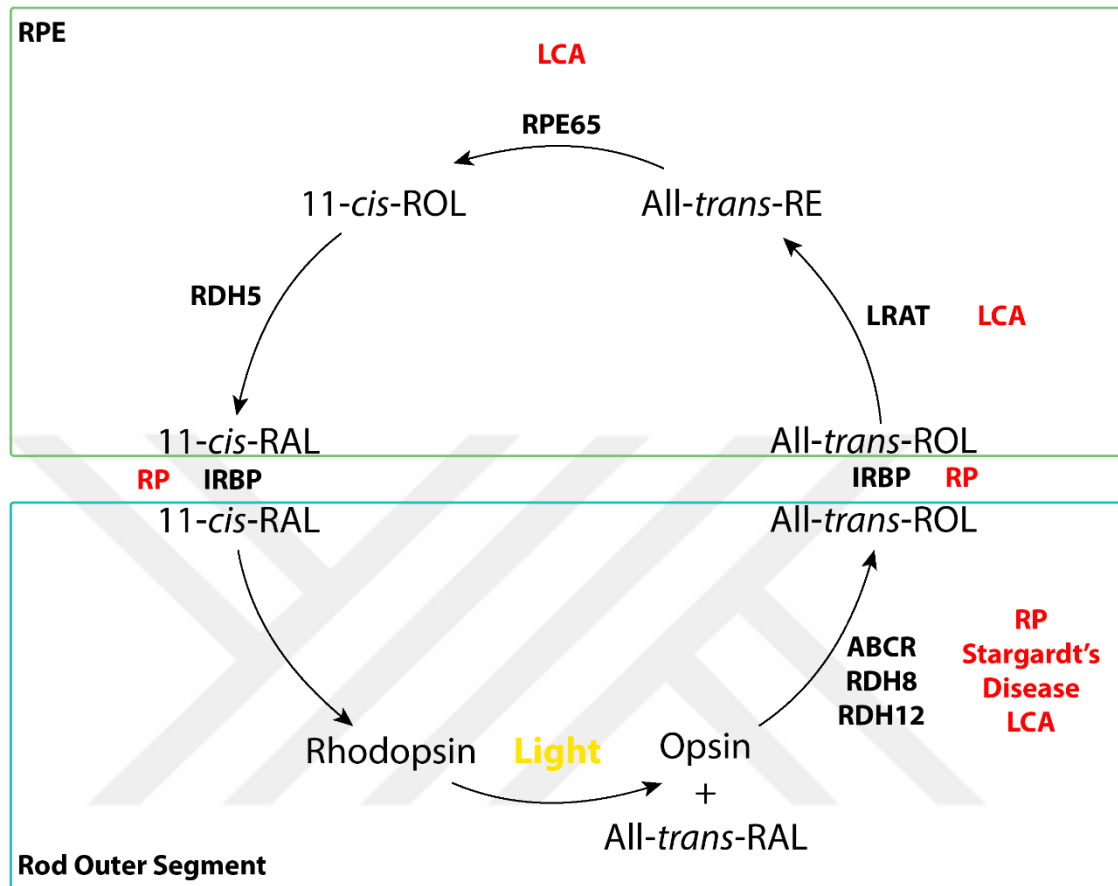


Figure 3. A diagram of the canonical visual cycle and relevant IRDs associated with key enzymes in the visual cycle. The visual pigment, opsin bound to 11-*cis*-RAL, receives light and goes through a conformational change where 11-*cis*-RAL transforms into all-*trans*-RAL. The downstream photosignal pathway is activated by the visual pigment and it breaks down into opsin and all-*trans*-RAL. All-*trans*-RAL is carried to the cytosolic side of disc membranes by ABCR and it is transformed into all-*trans*-ROL by RDH enzymes. IRBP transports the all-*trans*-ROL into the RPE, where it goes through esterification, catalyzed by LRAT, into all-*trans*-RE. All-*trans*-RE is then processed by the isohydromelase RPE65 enzyme into 11-*cis*-ROL. 11-*cis*-ROL is then transformed into 11-*cis*-RAL by RDH. IRBP carries the 11-*cis*-RAL into the photoreceptor discs, thus, allowing the visual pigment to regenerate. Diseases that are associated with the key enzymes in the visual cycle are denoted in red. Figure adapted from [23, 24].

Lastly, the photosignal is converted to electrical signal through a messenger molecule called cGMP. In dark conditions, the cGMP concentrations are regulated through guanylyl cyclase (GC) producing cGMP, while phosphodiesterase (PDE) hydrolyzing cGMP. A portion of the cGMP binds to and opens up cyclic nucleotide-gated (CNG) channels, allowing an inward current (dark current) of Na^+ and Ca^{2+} . The dark current keeps the photoreceptors depolarized, and in a state where they constantly release the

neurotransmitter glutamate. Activation of transducin by the visual pigment activates PDE, and causes rapid reduction of cGMP concentration. As a result, the dark current is blocked by the closure of CNG channels, and the photoreceptors become hyperpolarized. The inhibition of glutamate release conveys the photosignal to the postsynaptic neurons [22].

1.4. Inherited Retinal Diseases

The complexity of the retinal neuronal network and the visual cycle, and the high energy requirements of the retina makes it vulnerable to mutations in the key proteins of major retinal pathways. Due to these factors, devastating IRDs that are the leading cause of irreversible blindness such as non-syndromic RP, LCA and Usher syndrome are the focus of eye research. Monogenic IRDs are caused by rare genetic variations between individuals, thus, a single gene can have numerous causative mutations. Biomarker identification and candidate gene repertoire studies for genetically complex IRDs are being performed as well. However, a definitive cure for IRDs still do not exist. Currently, only viable medical strategy for IRDs is to treat the symptoms [25, 26]. Inheritance patterns of IRDs vary according to the location and number of mutations. A dominant inheritance pattern is where a loss-of-function (LOF) mutation in a single allele can result in haploinsufficiency, as the remaining allele is not sufficient for normal functioning. A second mutation in the other allele would be necessary for the recessive inheritance of a disease. Other possibilities for dominant inheritance pattern, such as gain-of-function (GOF) and dominant negative mutations, exist as well [26]. Thus, each IRD needs a specialized approach according to disease causing mutations. Retinitis pigmentosa and LCA, the leading cause of blindness in IRD patients, will be discussed in detail.

1.4.1. Non-Syndromic Retinitis Pigmentosa (RP)

RP is a group of diseases that is the most commonly diagnosed IRD. Prevalence of RP is estimated to be 1:4000. The progression of the disease and severity changes according to the disease causing mutation. Non-syndromic RP represents 65% of all RP cases [27]. RP inheritance patterns are autosomal dominant (ad), autosomal recessive (ad) or X-linked recessive (xlr). It is also possible for spontaneous occurrence of RP called simplex RP (sRP). The initial symptom is usually night blindness that is followed by gradual loss of peripheral vision. RP patients ultimately lose their visual capabilities over time. Photoreceptor layer gradually degenerates in RP patients due to apoptosis caused by mutations. RP is classified as a rod-cone dystrophy in which the progressive loss of rod photoreceptors is followed by degeneration of cone photoreceptors [27-30]. The cause of cone cell degeneration is not clear. It is suggested that the loss of survival factors released by rod cells and the increased oxidative stress can cause cone cell degeneration in RP [31, 32].

RP is a heterogeneous disease with more than 100 identified genes (RetNet, <https://sph.uth.edu/RetNet/>). *RHO* mutations are the leading cause of inherited RP. They are known to cause adRP, arRP, as well as congenital night blindness. It is estimated that 25-40% of all adRP patients have a disease-causing mutation in the *RHO* gene [28-30]. 50-60% of RP cases are caused by autosomal recessive mutations. P23H (Pro23His) is a well-studied and characterized *RHO* mutation that is mostly isolated to adRP patients in US [33]. It is a substitution mutation that replaces the highly conserved proline-23 with a histidine aminoacid. This class III *RHO* mutation impairs the retinal binding capability of opsin protein, and the visual cycle. P23H mutant protein can still bind to retinal albeit inefficiently [34]. The exact mode of action of P23H in RP disease is unclear. It was found that instability of P23H can cause unfolded protein response in transgenic rats. Disorganization of the ROS membrane structures were reported in *P23h* heterozygous and homozygous mice as well [35]. Furthermore, P23H RHO is retained and degraded in the endoplasmic reticulum. It is hypothesized that P23H RHO prevents the existing wild-type RHO from reaching the plasma membrane [36]. The mechanisms of P23H RHO cytotoxicity needs to be researched in further detail.

arRP is a disease group with more than 30 candidate genes. *RPE65* and *PDE6* mutations are estimated to cause 10% of all arRP cases. *RPE65* mutations inhibit the RPE65 protein function altogether, thus, impairing visual cycle and causing cytotoxicity [28]. *PDE6* mutations, especially *PDE6A* and *PDE6B*, cause high cGMP levels and the constant

opening of cation channels. Aberrant cation channel function causes constant Ca^{+2} influx and cell death [37]. Other missense (p.E150K [38], p.Met253Ile [39]), and nonsense (p.W161X [40], p.E249ter [41]) mutations were shown to be causing rare forms of arRP as well [42]. p.W161X and p.E249ter undergo nonsense-mediated decay, thus, these mutations can cause slow progressing arRP in homozygous forms, and they should not significantly affect visual acuity in heterozygous forms [43]. p.E150K is known to cause a mild version of arRP as well [44]. All in all, arRP causing mutations are understudied compared to adRP *RHO* mutations [42]. LCA is a rare IRD that is occasionally classified as a severe RP subtype. 1 in 30000 to 80000 people and 5% of all IRD patients are suffering from LCA. It is an early-onset severe rod-cone dystrophy that causes night blindness, and loss of vision that can result in complete blindness in severe cases. The onset of disease can be as early as 6 weeks of age. Oscillations of the eye is a crucial sign of LCA in children [23, 45]. 16% of LCA is caused by autosomal recessive mutations in the *RPE65* gene, classified as LCA2. *RPE65* mutations usually cause aberrant protein folding instead of directly modifying the protein active site. Thus, some *RPE65* mutations cause milder versions of LCA, where *RPE65* is somewhat functional, while others can cause complete loss of protein function (LCA2) [23, 46, 47].

1.4.2. Mouse Models of IRDs

1.4.2.1. P23H Mouse Line

Mouse models of IRDs were either generated or identified as spontaneous mutations by researchers. P23H mouse line (B6.129S6(Cg)-*Rho*^{tm1.1Kpal}/J, JAX#017628) was generated by Sakami et al. in 2011 [48]. Mouse P23H gene was cloned into an FRT-loxP-flanked neomycin knock-in targeting vector. The vector was transfected into iTL1 129 Sc/Ev embryonic cells, which were then injected to C57BL/6 blastocysts. Resulting animals were used to generate P23H homozygous animals, and the neomycin cassette was removed by crossing these animals to Cre recombinase expressing mouse line. Cre

recombinase transgene was eliminated by crossing as well. The resulting P23H knock-in animals have a 174-bp insert in the intron between exon 1 and exon 3. This insertion was used for PCR genotyping of P23H animals. P23H ki/wt mouse retinas had 20-40% thinner ONL (6-7 rows) compared to control (P23H wt/wt, 9-10 rows) animals at post-natal week (PNW) 9. P23H homozygous mice had lost almost all of the ONL by PNW 9. At PNW 16, P23H ki/wt mice had 4 rows of ONL in the ventral retina and 2 rows of ONL in the dorsal retina. This indicates an asymmetrical degeneration pattern in the mouse retina. P23H ki/wt mice had shortened ROS as early as PNW 5. While cone cells in P23H ki/wt mice were morphologically and numerically similar to their control counterparts, half of rod cells were degenerated by PNW 9. Electroretinogram (ERG) data also points to similar results. Scotopic ERG response is halved by PNDW 6 with slightly reduced photopic responses. However, both scotopic and photopic responses are significantly diminished by PNW 24. RHO protein levels were reduced 10-100 times in P23H ki/wt retinas compared to control retinas. P23H was retained in the ER and degraded due to incomplete glycosylation in P23H homozygote retinas. RHO was localized to the ROS in P23H ki/wt similar to wt retinas, indicating some of the P23H RHO could reach ROS. However, ROS lengths were much shorter in P23H ki/wt retinas. In closer inspection through electron microscopy, it was shown that P23H heterozygote ROS had perpendicular discs covered by plasma membrane. These aberrant structures cause ROS disorganization, and they can be the reason for retinal degeneration [48].

1.4.2.2. Rd10 Mouse Line

The Jackson Laboratory identified and characterized rd1, rd2, rd3, rd4, rd5, rd6, rd7, rd8, rd9, and rd10 retinal degeneration mouse models, each caused by different mutations [49]. Rd1 and rd10 animals contain different mutations in the *Pde6b* gene. *Pde6b* gene produces the β subunit of the PDE6 protein. Rd1 is a severe retinal degeneration model while rd10 presents with a comparably slower, but still fast, progression of retinal degeneration. *Pde6b*^{rd10} was first identified as a spontaneous missense mutation in a

CXB-1 recombinant mouse line due to retinal abnormalities. This results in cGMP accumulation and hinders cGMP signalling. *Pde6b^{rd10}* mutant CXB-1 line was backcrossed with C57BL/6J animals to produce the rd10 congenic mouse line. Degeneration of rod cells starts at postnatal day (PND) 16 and the whole ONL layer is degenerated by PND 60. Interestingly, rd10 animals kept in dark conditions had healthy retinas by PND 24, while this had no effect on rd1 retinas. Postnatal rd10 animals never display normal ERG responses. Compared to C57BL/6J, rod cell ERG responses were reduced to 10% by PND 30, while cone cell response was 30% at the time. Rod cell ERG responses were lost by PND 50 and cone cell responses were lost by PND 63 [49, 50]. cGMP-mediated toxicity is thought to be the main cause of retinal degeneration in rd10. *Cngb1^{-/-}* animals crossed to rd10 produces a *Cngb1* knockout rd10 line, without CNG channels, with a slower retinal degeneration rate. It was also shown that Pde6 expression and localization is impaired in rd10 mice. PDE6 subunits are found in reduced amounts throughout the rod cells in rd10 retina, as opposed to ROS localization in normal retina. PDE6B levels in rd10 retinas were 10% of that in C57BL/6 retinas [37].

1.5. Gene Therapy and Gene Editing Tools

The growing MSVI population and its economic burden put IRDs and other eye diseases on the focus of researchers around the world. IRDs are currently incurable by traditional

medicine. However, novel techniques with incredible therapeutic potential were discovered in the last decades. These techniques brought about gene therapy, a

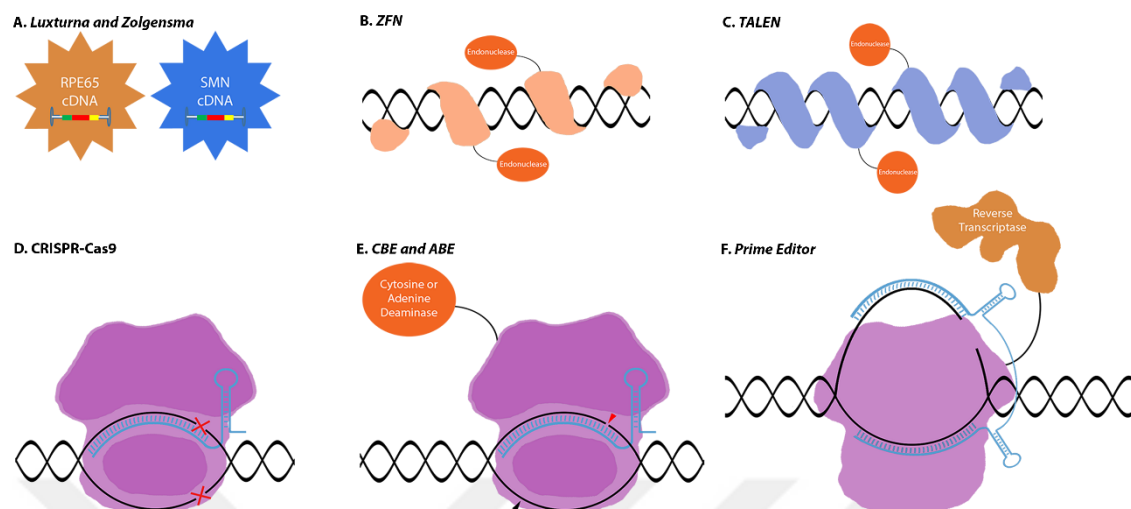


Figure 4. Commonly used gene replacement and gene correction tools. A) Viral delivery of wild-type cDNA is successfully used for the gene replacement therapy of RP, LCA and spinal muscular atrophy (SMA). B-D) ZFN, TALEN and CRISPR-Cas9 are gene can be used for gene replacement as well as gene correction therapies. CRISPR-Cas9 in combination with NHEJ and HDR can enable mutation correction in vivo. E, F) Novel gene correction toolset that is based on CRISPR-Cas9 system and its variants. While CBE and ABEs use Cas9n for efficient correction of SNP variants, prime editors use Cas9n for correction of a wide variety of mutation including SNP, insertions, and deletions. Figure taken from [51].

clinical therapy method that aims to cure or treat a disease by using recombinant nucleic acids or proteins, that alter the genome or the transcriptome of an organism. Gene therapy gained worldwide popularity following the approval of voretigene neparvovec (VN, Luxturna) by U.S. Food and Drug Administration (FDA) [11], and European Medicines Agency (EMA) [52]. VN is a gene therapy product available to RPE65-related RP and LCA2 patients. VN delivers the wild-type cDNA of the *RPE65* gene by subretinal AAV2 injections. VN increased the multi-luminance mobility test scores of patients, signifying the increase in their ability to navigate in varying light conditions. The light sensitivity, and expectedly, the peripheral vision of patients increased after VN treatment. No immune-related adverse events were reported due to VN injections [10, 53]. Recently, retinal atrophy around the subretinal bleb, the area around the subretinal injection site, was indicated after subretinal VN injections. However, patients had retained or improved visual acuity, mainly due to fovea remaining unaffected [54]. VN is a long-term cost-effective therapy that is now the main treatment option for people with RPE65-related diseases [55]. The success of VN showed that gene therapy is a viable alternative to

traditional medicine. VN is delivered by an AAV2 and it directly delivers the healthy cDNA of the *RPE65* gene, classifying it as a gene replacement therapy. However, this method is not readily available for most of the IRDs. Advanced forms of gene therapy products were developed to overcome the shortcomings of gene replacement therapy. Gene therapy is achieved by the delivery of a gene editing tool, in a targeted or untargeted manner, into an organism. Gene therapy relies on a carrier/delivery tool, and a gene editing tool or the wild-type cDNA of the target gene. Overall, gene therapy relies on gene editing tools to either 1) directly replace the mutant gene by gene replacement, 2) correct the mutations in the disease-causing gene by gene correction, or 3) regulate the expression of genes in relevant pathways to alleviate and treat the disease symptoms.

1.5.1. Adeno-Associated Virus (AAV)

AAVs are small (4,7-5 kb), linear ssDNA viruses from the Parvoviridae family and *Dependovirus* genus [56, 57]. They were initially identified to be contaminating adenovirus (Ad) isolates. They require coinfection by a helper virus, such as Ad or a herpesvirus [57]. 90% of the adults in US are estimated to be seropositive for AAV. However, AAVs are shown to be nonpathogenic. Wild-type AAV can integrate into the host genome, nonetheless, recombinant AAVs (rAAVs) are designed to stay episomal and be replication-defective [56]. The *rep*, *aap*, and *cap* genes that are flanked by inverted terminal repeats (ITRs) at 5' and 3' ends make up the wild-type AAV genome. Rep78, Rep68, Rep52, and Rep40 are non-structural proteins that are expressed by the *rep* gene and are necessary for viral replication and integration into the host genome, but they are insufficient to create a mature AAV particle. The VP1, VP2, and VP3 proteins encoded by the *cap* gene are used in a 1:1:10 ratio to form the icosahedral capsid of AAVs. AAP protein is not necessary for the assembly of several AAV capsids but is essential for the nucleolar localization of the VP proteins during the assembly of AAV2 serotype [58]. In the presence of a helper virus, the AAV goes into lytic cycle and reproduces itself. Lack of a helper virus causes the AAV to either stay episomal and form DNA concatamers, or integrate its genome into the host [59]. To date, 13 different AAV serotypes have been discovered. The most prevalent AAV serotype, AAV2, uses heparan sulfate

proteoglycans to perform the initial attachment to the cell surface. AAV2 shows tropism for a large population of tissues such as brain, eye, liver and muscles. AAV5 is known to show tropism towards photoreceptor cells and liver tissue while AAV8 shows muscle tissue tropism [60-63]. The whole AAV genome other than the 5' and 3' flanking inverted terminal repeats (ITRs) can be replaced in recombinant AAV vectors. The transgene region should not exceed approximately 4.7kb, the packaging capacity of AAVs, to avoid reduced AAV transduction efficiencies and truncated delivery of the vector. The small packaging capacity, and the AAV antibody induced immunogenicity are the limiting factors of an AAV-mediated gene therapy [58, 64, 65]. Furthermore, although AAVs show tropism towards specific tissues, they can still transduce other cell types. Thus, *in vivo* delivery of AAVs into the bloodstream can result in AAV accumulation in the liver [58]. Directed evolution is used for generation of AAV capsids that specifically target a cell population. As Müller cells are shown to have a direct connection to neuroprotection in retina, they were targeted through directed evolution to generate an AAV serotype called AAV2/ShH10. AAV2/ShH10 was derived from AAV6 and it efficiently transduced rat Müller cells [66].

1.5.2. Gene Editing and Regulatory Tools

Eye diseases occupy a big portion of the currently active clinical trials that are using gene therapy products. It was shown that eye diseases account for 8% of the gene therapy clinical trials and the eye is the only organ with such focused research on gene therapy. Most of these gene therapy products rely on gene replacement. However, several clinical trials point to a new generation of gene therapy products that use novel gene editing tools [51]. These new generation of gene editing tools are based on antisense oligonucleotides (ASOs), zinc finger proteins (ZFPs), transcription activator-like effector (TALE) proteins, and CRISPR-Cas systems.

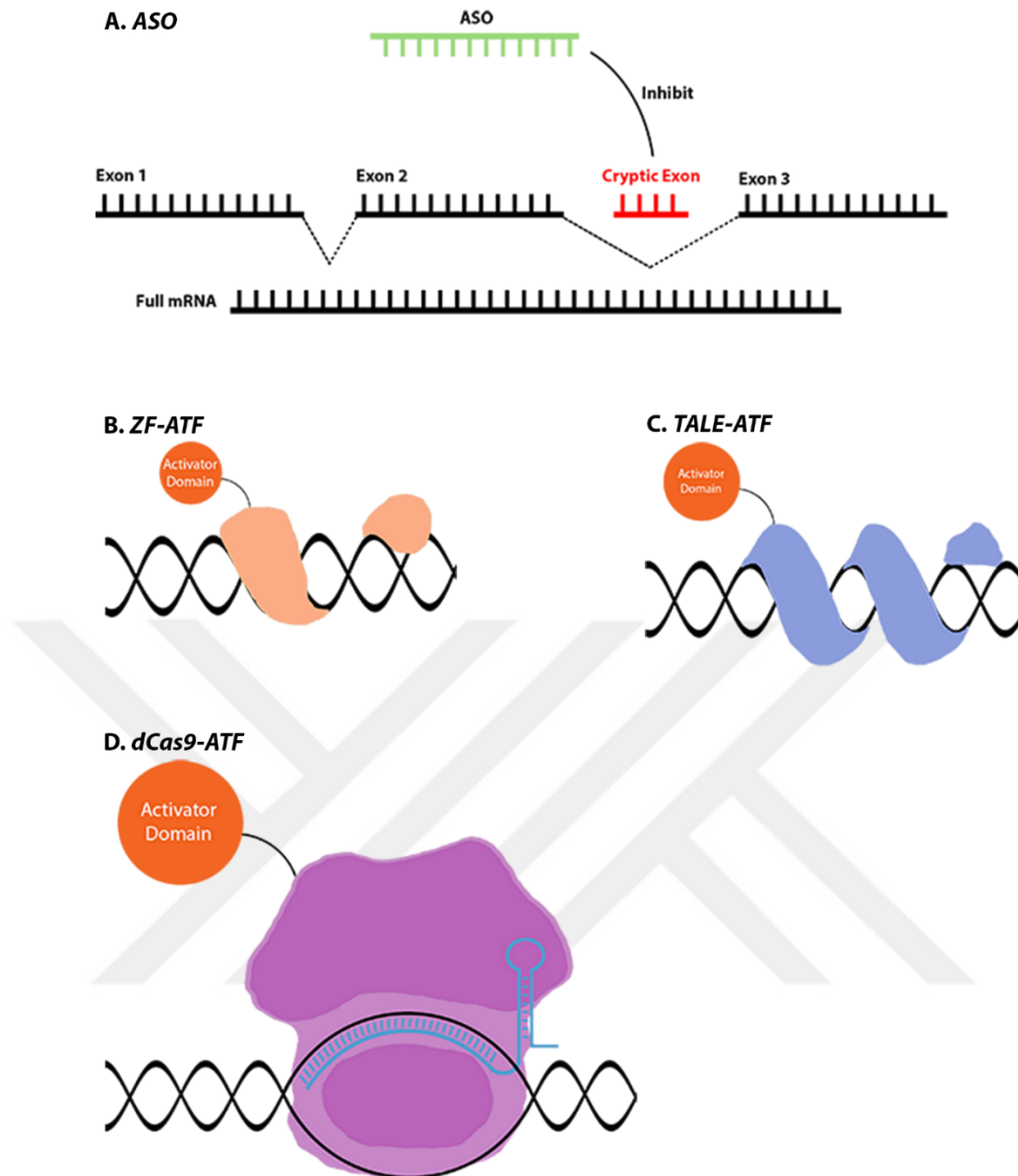


Figure 5. Gene regulatory tools for altering the transcriptional profile of genes. *A)* ASOs are one of the older gene regulatory tools that is widely used for retinal as well as other diseases. Their main uses are gene silencing and modulation of mRNA splicing. *B, C)* ZF-ATF and TALE-ATFs are gene editing tools that achieve transcriptional regulation through a protein:DNA interaction. They are rather complex and costly compared to alternatives with similar functionalities. *D)* dCas9-ATF uses a gRNA to target specific genes and regulates their expressions. dCas9-ATFs are versatile tools that are easily modifiable. Figure taken from [51].

Zinc finger proteins were identified in *Xenopus* oocytes in 1980. It was a transcription factor that was crucial for the transcription of 5S RNA. It was discovered that this transcription factor contained zinc ions that were necessary for DNA binding. Moreover, most of the protein was made of 9 tandem repeats that were highly similar in sequence. Each of these repeats were named zinc fingers (ZFs) [67]. ZFPs are highly modular, modifiable, and specific. Each ZF targets 3-4 DNA base pairs and a ZFP module can be

engineered to target specific base modifications [68]. Later, ZFPs fused to FokI endonuclease domains were developed, called ZF nuclease (ZFN), to produce double-stranded breaks (DSBs) in the genome. The DNA repair mechanisms non-homologous end joining (NHEJ) and homology-directed repair (HDR) then repair the damaged region. This technique can inhibit gene expression due to NHEJ-mediated mutations or replace a mutant gene by the delivery of homology arms that are recombined to the genome by HDR [69-71]. It is also possible to regulate gene expression using ZFPs. Gene expression can be blocked by extensively targeting the coding sequence or the promoter region of a gene. Another option is to make ZF fusion proteins with inhibitory (such as KRAB) or activatory (such as VP64, VPR, SPH) domains [68]. TALE is another gene editing tool that is inherently modular. TALE was first identified in *Xanthomonas sp.*, a plant pathogen. This pathogen modifies the transcriptome of the plant by using TALE as a transcriptional factor. Wild-type TALE proteins contain tandem repeats, nuclear localization signals and an activation domain. Each repeat recognizes a DNA bp and they contain 2 hypervariable regions, called repeat variable diresidues (RVDs), that are responsible for the specificity of the monomer [72, 73]. Similar to ZFNs, TALEs can be fused to endonuclease, activatory or inhibitory domains. TALE proteins can target virtually any sequence in the genome as well. Furthermore, TALEs provide a cheaper, more available alternative to ZF, due to the intellectual property rights required for the highly researched ZF techniques [72]. ZFNs and TALENs were the first generation of gene correction tools that could potentially correct mutations in any sequence of the human genome, however, their use in eye diseases remained very low. The complexity of the cloning and production of these systems made them proportionally expensive. ZFs and TALE proteins are still used in research to develop gene regulatory tools called artificial transcriptional factors (ATFs) [74].

A new set of gene editing tools was born with the discovery of CRISPR-Cas systems. CRISPR-Cas9 is an RNA-guided DNA-targeting system that produces DNA DSBs near short conserved sequences called protospacer-adjacent motifs (PAMs). CRISPR-Cas9 system was first isolated from the bacteria species *Streptococcus pyogenes*. The endonuclease activity depends on the complementarity between the guide RNA (gRNA) and the target sequence [75]. Wild-type CRISPR-Cas systems are prokaryotic defense mechanisms, similar to the adaptive immune system, against bacteriophages. Snippets of invading bacteriophage DNA would be cut and inserted into the hosts genome adjacent

to the CRISPR locus. These small DNA would then be used to target the invading bacteriophage genome, in which CRISPR-Cas9 produces DSBs. Cas9 protein contains two endonuclease domains, called HNH and RuvC, that cleaves the DNA. This targeting system is achieved by CRISPR RNAs (crRNAs) and *trans*-activating CRISPR RNAs (tracrRNAs). crRNAs are responsible for targeting while tracrRNA plays a role in crRNA processing and DNA cleavage by Cas9. crRNA and tracrRNA were combined to form a single guide RNA (sgRNA) by researchers [75-77]. The sgRNA can be customized to target any sequence in the genome that is flanked by a PAM sequence. Thus, only the sgRNA is produced for each target in the CRISPR-Cas systems, whereas the whole ZFN or TALEN protein needs to be produced for each target. This property alone makes CRISPR-Cas systems considerably cheaper and simpler to produce than ZFNs and TALENs as only the gRNA. CRISPR-Cas9 was used to correct a CEP290 mutation that causes LCA subtype LCA10. An AAV5-SaCas9 system was delivered subretinally and 21% deletion of the mutation was achieved *in vivo* [78]. This system is now undergoing a clinical trial (NCT03872479) following the positive results from their preclinical studies. AMD is another eye disease that is a focus of gene therapy research. A CRISPR-SpCas9 system targeting the vascular endothelial growth factor (*Vegfa*) loci was developed for the treatment of neovascularization in AMD. *Vegfa* targeting SpCas9 (mLP-CRISPR) was delivered by a lentivirus and it achieved 35% decrease of its expression in the outer layers of retina (RPE-choroid-sclera) *in vivo*. Furthermore, mLP-CRISPR reduced the neovascularization area by 63% in a neovascular AMD mouse model [79].

The simple but advanced gene editing tool CRISPR-Cas system was researched intensively to identify and characterize novel CRISPR-Cas variants. Furthermore, CRISPR-Cas fusion proteins with specific properties were produced. For example, the base editor cytidine deaminase was fused to CRISPR-Cas9 to produce targeted point mutations that would convert C and G to T and A base pairs, respectively [80]. This system, called cytidine base editor (CBE), was later enhanced by replacing Cas9 with a Cas9-nickase (Cas9n) that has a single active endonuclease domain, and fusing uracil glycosylase inhibitor (UGI) to the C-terminal of Cas9n. Cas9n nicks the opposite strand of the U produced by the CBE to increase the base editing efficiency. Inclusion of the UGI prevents base excision repair mechanism that is initiated by the uracil N-glycosylase from excising the edited U [81]. Adenine base editor (ABE) was produced by fusing a

recombinant *E.coli* adenine deaminase TadA to the Cas9n. A TadA protein that efficiently targets DNA was produced through directed evolution. The resulting ABE successfully converts A and T base pairs to G and C [82]. A more advanced CRISPR-Cas-based gene editing tool called prime editor was developed recently. Prime editor uses a special gRNA called prime editing guide RNA (pegRNA) and a fusion protein of Cas9n and a Moloney murine leukaemia virus (M-MLV) reverse transcriptase (RT). pegRNA is used for DNA targeting and it forms heterodimers with the nicked DNA strand to introduce a reverse transcribed sequence into the target site. Prime editing systems are less constrained by PAM sequence requirements while ABE and CBE systems have higher efficiency in target sites closer to the PAM sequence [83]. CRISPR-Cas9, ABE, CBE, and prime editors are gene editing tools that are used for gene correction therapy. This novel toolset offers the correction of disease-causing mutations such as single nucleotide variants and indels.

Gene regulatory tools achieve the upregulation or downregulation of a gene by either using a *cis*-acting transcriptional regulator domain (ATF), or through DNA/RNA antisense mechanisms (ASO). ASOs are short DNA sequences that are designed to complement mRNA sequences to either block the expression of a gene or modify the splicing of an mRNA. ASOs perform knockouts by producing a DNA:mRNA heteroduplex which gets cleaved by RNase H [84]. Uteversen, IONIS-FB-L_{RX}, Aganirsen, Sepofarsen and QR-1123 are ASO drugs targeting eye diseases and they are currently in clinical trials. Aganirsen inhibits the insulin receptor substrate I (IRS-1) expression, which was shown to downregulate *VEGF* expression as well [85]. Inhibition of *VEGF* is crucial in ischemic retinal vein occlusion and neovascular glaucoma, in which aberrant leaky vessel formation causes retinal degeneration [86]. Aganirsen was shown to reduce neovascularization in animal models [87] and it is actively in a clinical trial (NCT02947867) [88, 89]. IONIS-FB-L_{RX} is an ASO drug undergoing clinical trials for the treatment of atrophic AMD. It inhibits the complement factor B, and indirectly the alternative complement activation pathway [90]. Complement system activation has been implicated in AMD in the literature [91]. IONIS-FB-L_{RX} is in clinical trials as well (NCT03815825, NCT04014335). ASOs modifying mRNA splicing are used in therapeutics targeting IRDs as well. Uteversen is an ASO drug that enables the skipping of exon 13 of *USH2A* gene. *USH2A* gene has been known to cause arRP and Usher syndrome type 2 (USH2). *USH2A* mutations cause 12-25% of arRP and 50% of all USH2

cases. USH2 is a syndromic disease with moderate-to-severe hearing loss followed by retinal degeneration [92]. Preclinical tests of Uteversen were performed with successful results [93], and it is undergoing clinical trials (NCT05158296, NCT03780257, NCT05085964, NCT05176717). As mentioned before, P23H RHO might have a role in preventing wild-type P23H functionality [36]. QR-1123 is an ASO therapeutic that was developed to inhibit targeted knockout of P23H RHO expression. QR-1123 received fast-track designation from the FDA and is in a clinical trial [94] (NCT04123626).

ATFs, similar to ASOs, are gene regulatory tools that modify the existing transcriptional network in cells for the treatment of a disease. CRISPR-Cas system was modified to produce a catalytically inactive form of Cas9 called CRISPR-dCas9. CRISPR-dCas9 was purposed for transcriptional regulation as an ATF. dCas9 alone can silence gene expression by binding to the promoter region or the gene itself [95]. CRISPR-dCas9 system was fused to activatory (such as VP64, p65, Rta, VPR) and inhibitory domains (such as KRAB), and their *in vitro* uses were successfully shown [96, 97]. VP64 is an activator domain that consists of four copies of the minimal activation domain of the VP16 from the Herpes simplex virus [98]. P65 is a subunit of the NF- κ B protein that acts as a transcriptional activator [99]. Rta, Epstein-Barr virus R transactivator, is a transcriptional activator that was first isolated from the Epstein-Barr virus [100]. VPR is a fusion protein that combines the transcriptional activator domains VP64, p65 and Rta to form the transcriptional activator VPR [96]. dCas9-VPR was shown to transactivate cone photoreceptor medium wavelength opsin (*Opn1mw*) *in vitro* and *in vivo*. Transactivation of *Opn1mw* in the rod cells of an RP mouse model with a heterozygous RHO mutation increased the ONL thickness and ERG responsiveness of the treated animals [101]. SunTag was developed as a precise signal amplification system that utilizes the GCN4 transcriptional activator [102, 103]. SunTag is comprised of GCN4 repeats that are bound to a target protein, and a GFP protein bound to a single chain variable fragment (scFv) antibody that targets GCN4. This imaging system enabled highly precise intracellular imaging [103]. SunTag was used in dCas9-ATFs, in which GCN4 repeats were fused to dCas9 and an scFv-VP64 was delivered [104]. The VP64 in SunTag was replaced by p65-HSF1-EGFP to produce the dCas-SPH transcriptional activation system [105]. HSF1, human heat-shock factor 1, has various roles in other than the heat-shock response, and it was shown that HSF1 improves transcriptional activation efficiency of dCas9-ATF [106]. dCas-SPH vector contains LSL upstream to the dCas9

and 10xGCN4 that is P2A linked to the scFv-p65-HSF1-EGFP downstream. Thus, dCas-SPH expression is Cre-dependent. dCas-SPH vector contains piggyBac ITR sites flanking the 5' and 3' ends. This dCas-SPH-piggyBac vector was coinjected with a piggyBac transposase vector into zygotes. The dCas-SPH system was shown to successfully overexpress genes and be capable of multiplex overexpression of genes.

1.6. Neuroprotection in Retina

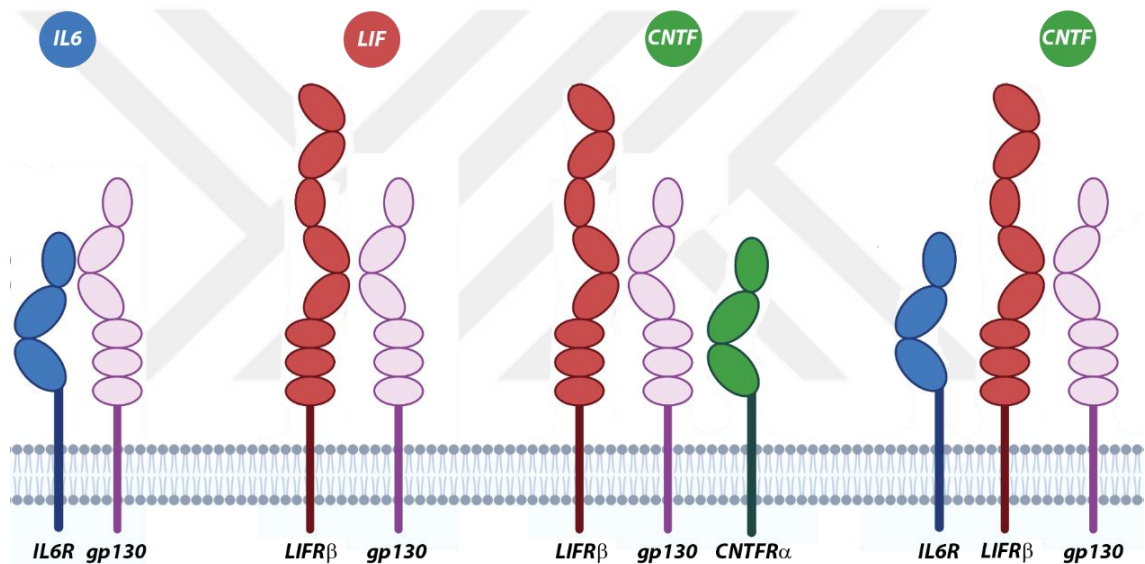


Figure 6. IL-6 cytokine family receptors and their different combinations. IL-6 itself interacts with IL6R and gp130 to produce a hexamer of 2:2:2 ratio of each protein. LIF uses the LIFRβ and gp130 receptor complex for signalling. CNTF can interact with either the LIFRβ, gp130, CNTFRα or the IL6R, LIFRβ and gp130 tripartite receptor complexes [107]. Figure adapted from [107] and created with BioRender.com.

The etiology of IRDs are extensively researched with hundreds of candidate genes identified and classified. However, the exact mechanisms of cell death in IRDs such as RP and LCA remain uncertain. IRDs can be monogenic and simple in terms of disease genetics. On the other hand, different mutations in the same gene can cause vastly different disease progressions (such as *USH2A*). The endpoint of IRDs being loss of photoreceptor cells remains the same between all of them. Thus, neuroprotection is critical for retinal degenerative diseases to protect cells against apoptosis [108]. Neurotrophic factors are a group of proteins that can alleviate the progression of retinal degeneration. They are secreted by central nervous system (CNS) neurons as well as the

retina, which is an extension of the CNS. Some of the important neurotrophic factors expressed in the CNS are VEGF, insulin-like growth factor (IGF), basic fibroblast growth factor (FGF2), ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), leukemia inhibitory factor (LIF), and mesencephalic astrocyte-derived neurotrophic factor (MANF) [109, 110].

CNTF and LIF are part of the interleukin-6 (IL-6) cytokine family. CNTF and LIF share structural similarities with each other and IL-6, as they all have a long-chain four-alpha-helix bundle. IL-6 family of cytokines all share the receptor complex subunit gp130. However, their interactions with their corresponding receptor complexes are different. For example, blocking the IL-6 interaction site on gp130 does not block LIF signal transduction [107]. IL-6 family also shares gp130 and JAK-STAT signalling pathways. LIFR β induces SHP-2-MAPK while CNTF and LIFR β induces transient STAT3 signalling pathways. LIFR β and CNTF induced STAT3 signaling is unsustainable due to the ERK and SOCS3 negative feedback [107, 111]. LIFR β is overexpressed following inflammation and CNTF deficiency causes earlier onset of multiple sclerosis as well [107]. CNTF was shown to increase RGC and photoreceptor cell survival in retinal degeneration models [112, 113]. However, increased expression of CNTF can cause morphological changes in rod cells. It was discovered that excess delivery of CNTF to the retina causes lowered ERG responses until the CNTF levels return to basal expression levels. This effect of CNTF might also be neuroprotective as reduced light sensitivity proportionally reduces the energy requirements of photoreceptor cells [112]. LIF expression is also required for a neuroprotective response in the degenerating retina. Müller cells are crucial in neuroprotection as they cause the neuroprotective gliosis reaction and express many of the neurotrophic factors, including LIF. LIF expression is recently found to induce the Wnt/ β -Catenin pathway, that has a neuroprotective effect in retina [114]. LIF is required for STAT3, Jak3 and FGF2 expression as well. LIF knockout mice showed a severe retinal degeneration phenotype due to the absence of cytokines downstream to LIF [115]. FGF2 is a cytokine induced by LIF and it activates the ERK pathway in addition to the common IL-6 pathways. Increased FGF2 expression has neuroprotective effects in rat retinal degeneration models. FGF2 overexpression protects photoreceptors against hyperoxia-induced damage in mice as well [116, 117]. Intravitreal delivery of FGF2 improved visual acuity in humans as well [118]. MANF is another neurotrophic factor that is important for neuroprotection. Its classification as a

neurotrophic factor is debated as it is expressed in other cell types and its deficiency results in diabetes. MANF alleviates the NF- κ B activation due to TNF α . MANF is retained in the endoplasmic reticulum and it is involved in the unfolded protein response (UPR) pathway. MANF was shown to downregulate UPR responses and increase cell viability under stress conditions. Furthermore, MANF interacts with NF- κ B p65 to reduce its activity and produces an anti-inflammatory effect [109, 119]. IL-6 family cytokines CNTF and LIF might have immunomodulatory effects and they are potential targets for neuroprotection in the CNS. FGF2 is shown to increase photoreceptor survival while MANF alleviate the inflammatory and UPR responses. Thus, neurotrophic factors are ideal targets for transcriptional regulation to attenuate the aberrant morphology in retinal diseases.



2. MATERIALS AND METHODS

2.1. Animal Breeding Strategy

All experiments on animals were approved by and under the supervision of Sabanci University and Gebze Technical University Ethics Committees. Animal experiments were designed according to the Association for Research in Vision and Ophthalmology's statement on the use of animals in research. Animals were kept in Gebze Technical University for the whole duration of this study. Animal maintenance records were stored using PyRat Animal Facility Software (<https://www.scionics.com/pyrat.html>). All mice were kept on a 12-hour light/dark cycle with ad libitum food and water. Rhodopsin P23H mutation B6.129S6(Cg)-*Rho*^{tm1.1Kpal}/J (Rho.P23H, Jax Strain #017628), Cre-dependent CRISPR-SpdCas9-SPH B6;D2-Gm33925^{Tn(pb-CAG-cas9*, -EGFP)1Yang}/J (dCas-SPH, Jax Strain #031645) transgenic mouse lines were back-crossed with C57BL/6J (Jax Strain #000664) for maintenance.

2.1.1. Rod Reporter and P23H Rod Reporter Mouse Lines

A rod reporter mouse model was previously generated in our lab by crossing B6.Cg-*Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}* (Ai14, Jax Strain #007914) and *Tg(Rho-cre)(Tg(Rho-Cre))*, Gifted by Yun Z. Le [120]) transgenic mouse lines. The produced rod reporter transgenic animals were crossed twice to obtain Ai14 homozygous offspring. Rod reporter animals homozygous for Ai14 were crossed with *Rho^{P23H/wt}* homozygous animals to obtain the P23H heterozygous rod reporter mouse line (P23H rod reporter, B6.Cg-*Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze/J}* x *Tg(Rho-cre)*). *Rho^{P23H/wt}* animals will be referred as P23H from this point on.

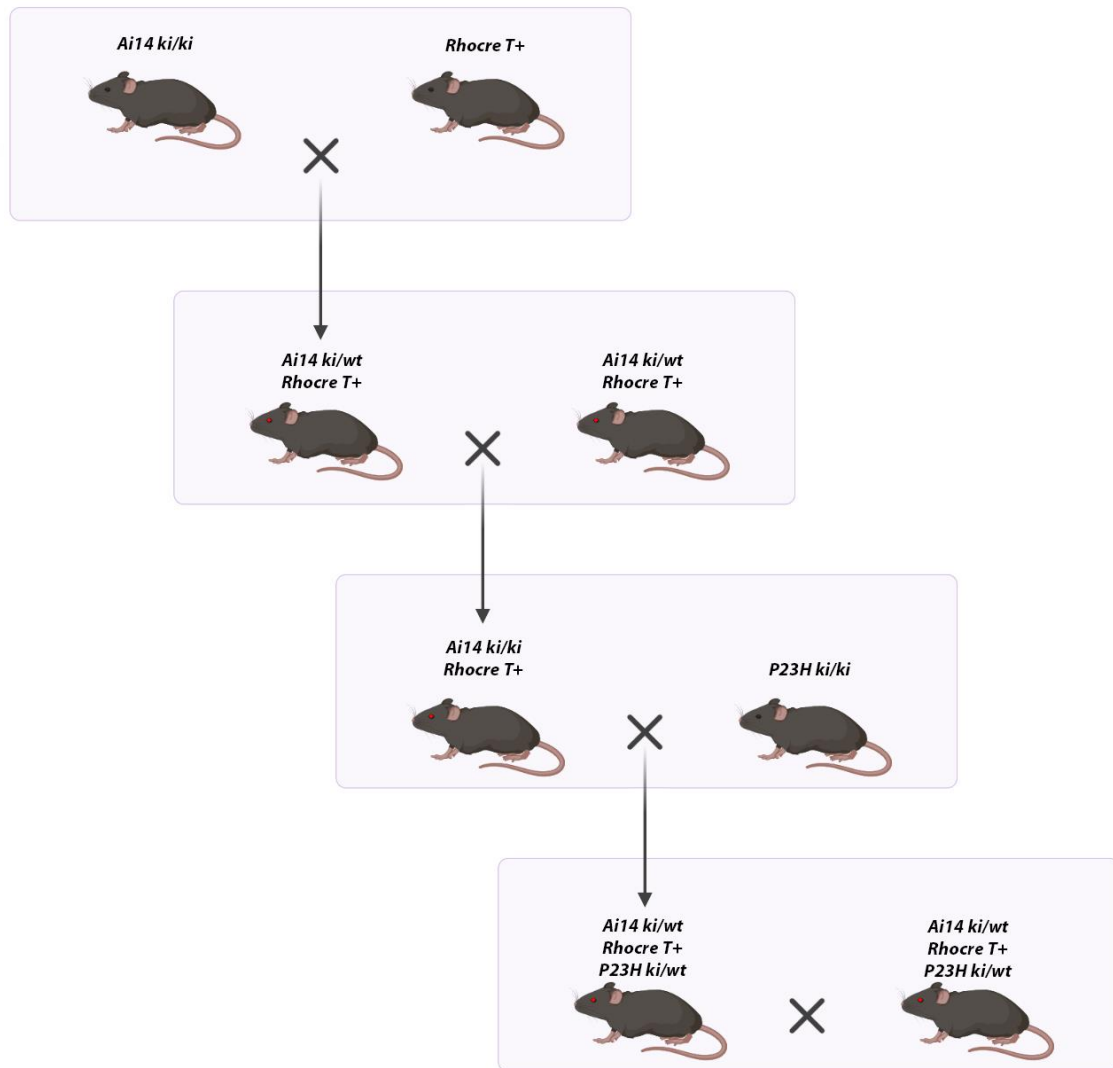


Figure 7. Breeding strategy for the rod reporter and P23H rod reporter mouse models. An Ai14 homozygous animal was crossed with a *Tg(Rho-Cre)* transgenic animal to obtain the rod reporter mouse model with Ai14 and *Tg(Rho-Cre)* transgenes. This F1 generation was inbred to obtain Ai14 homozygous rod reporter animals. These animals were crossed with P23H homozygous animals to obtain the P23H rod reporter mouse model that is P23H heterozygous and Ai14, *Tg(Rho-Cre)* transgenic. Figure created with BioRender.com.

2.1.2. P23H-SPH Mouse Line

During the development of P23H reporter mice, some animals lacking the Tg(Rho-Cre) transgene, but with P23H^{ki/ki} and Ai14^{ki/ki} mutations, were crossed with dCas-SPH animals to produce a slow retinal degeneration mouse model with P23H^{ki/wt} genotype that was used for *in vivo* dCas9-SPH-mediated neuroprotection study. These animals also expressed Ai14 that enables the tracking of Cre recombinase activity.



Figure 8. Breeding strategy for the P23H-SPH mouse model. The P23H reporter line was crossed with dCas-SPH mice to obtain an F1 generation that is heterozygous for P23H, Ai14, dCas-SPH transgenic and lacks Tg(Rho-Cre). The F1 generation was inbred to obtain P23H-SPH animals that are P23H heterozygous, Ai14, and dCas-SPH triple transgenic. Figure created with BioRender.com.

2.1.3. Rd10-SPH Mouse Line

Rd10 animals were crossed with dCas-SPH animals to produce a fast retinal degeneration model that is suitable for CRISPR-based neuroprotection studies. Generated Rd10-SPH (B6.CXB1-Pde6b^{rd10}/J x B6;D2-Gm33925^{Tn(pb-CAG-cas9*, -EGFP)1Yangh}/J) animals were inbred to retain the rd10 homozygous genotype.

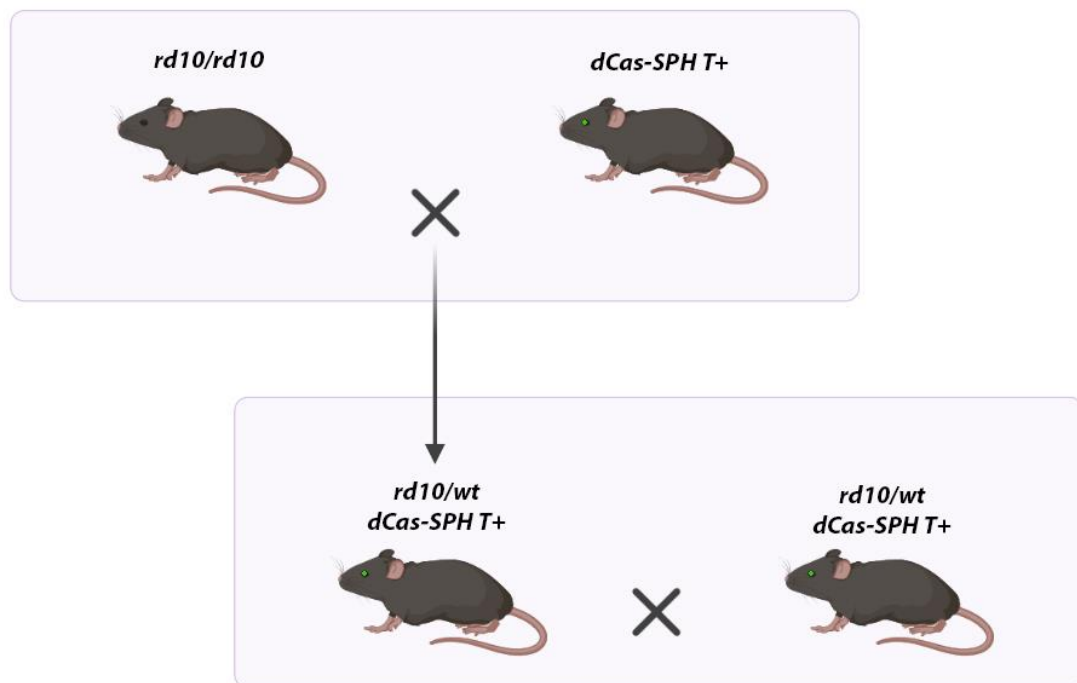


Figure 9. Breeding strategy for the rd10-SPH mouse model. A rd10 homozygous animal was crossed with a dCas-SPH transgenic animal to obtain the rd10 heterozygous, dCas-SPH transgenic rd10-SPH mouse model. Figure created with BioRender.com.

2.2. Genotyping

2.2.1. Toe Biopsy

Toe biopsy was performed at PND 5 to isolate DNA from transgenic mice tissue. 50 µl tissue lysis buffer was added to the isolated tissues. Then, tissues were incubated at 95°C for 90 minutes. Lysed tissues were neutralized by adding 50 µl of tissue neutralization buffer. Resulting samples were stored at -20°C.

Table 1. Tissue lysis buffer. Components of tissue lysis buffer and their concentrations.

Tissue Lysis Buffer		
Components	Volume (µl)	Final Concentration
Sodium Hydroxide	62,5	25 mM
EDTA	10	0,2 µM
Water	up to 25 ml	

Lysed tissues were neutralized by adding 50 µl of tissue neutralization buffer. Resulting samples were stored at -20°C.

Table 2. Neutralization buffer. Components of tissue neutralization buffer and their concentrations.

Tissue Neutralization Buffer		
Components	Volume (ml)	Final Concentration
Tris-HCl	1	40 mM
Water	24	

2.2.2. Polymerase Chain Reaction (PCR)

GoTaq green PCR reaction buffer (Promega, M7911) was used for the preparation of a common PCR mastermix.

Table 3. GoTaq Green Master Mix. Preparation of GoTaq Green master mix.

GoTaq Green Master Mix		
Components	Volume (ml)	Final Concentration
GoTaq Green Reaction	1	1X
Buffer		
MgCl ₂	0,3	2 mM
DMSO	0,2	1/21 (v/v)
dNTP	0,2	0,12 mM
Water	2,5	

Taq DNA polymerase (NEB, M0267L) was used for standard PCR reactions. PCR genotyping was performed using toe biopsy tissues. 2 µl of sample was added to each PCR reaction. All the components of PCR reaction mixture were added into a 0,2 ml PCR tube (Sarstedt, 72.737.002) to be vortexed and spun. PCR tubes were placed in a thermal cycler (Biorad, C1000 Touch Thermal Cycler) and an appropriate PCR protocol was used from **tables 5-7**.

Table 4. Standard PCR reaction. Preparation of the standard PCR reaction mixture.

Standard PCR Reaction		
Components	Volume (µl)	Final Concentration
GoTaq Green Master Mix	21	
Taq Polymerase	0.15	0,06 U/µl
Forward Primer	1	50 nM
Reverse Primer	1	50 nM
DNA	1-2	
Nuclease-Free Water	fill up to 25	

Table 5 was used for Ai14 wt and Ai14 mutant genotyping PCR reactions. **Table 6** was used for dCas-SPH and P23H genotyping PCR reactions. **Table 7** was used for Rd10 genotyping PCR reactions.

Table 5. Ai14 PCR protocol. PCR protocol used for Ai14 genotyping.

Ai14 PCR Protocol				
1	Initial Denaturation	94°C	2 min	
2	Denaturation	94°C	20 sec	
3	Annealing	65°C	15 sec	-0.5°C/cycle
4	Synthesis	72°C	10 sec	
Go back to step 2 for 9 times				
5	Denaturation	94°C	15 sec	
6	Annealing	60°C	15 sec	
7	Synthesis	72°C	10 sec	
Go back to step 5 for 28 times				
8	Elongation	72°C	1 min	
9	Cool down	4°C	hold	

Table 6. dCas-SPH PCR protocol. PCR protocol used for dCas-SPH and P23H genotyping.

dCas-SPH PCR Protocol				
1	Initial Denaturation	94°C	2 min	-0.5°C/cycle
2	Denaturation	94°C	20 sec	
3	Annealing	65°C	15 sec	
4	Synthesis	68°C	10 sec	
Go back to step 2 for 9 times				
5	Denaturation	94°C	15 sec	
6	Annealing	60°C	15 sec	
7	Synthesis	72°C	10 sec	
Go back to step 5 for 28 times				
8	Elongation	72°C	1 min	
9	Cool down	4°C	hold	

Table 7. Rd10 PCR protocol. PCR protocol used for Rd10 genotyping.

Rd10 PCR Protocol			
1	Initial Denaturation	94°C	5 min
2	Denaturation	94°C	30 sec
3	Annealing	61°C	45 sec
4	Synthesis	72°C	45 sec
Go back to step 2 for 35 times			
5	Elongation	72°C	10 min
6	Cool down	4°C	hold

2.2.2.1. P23H PCR

Using the primer set provided by The Jackson Laboratory (**table 20**), it was possible to identify heterozygous and homozygous animals apart. PCR bands show up at 320 bp for mutant animals while 150 and 320 bp for heterozygous animals, and 150 bp for wild type animals.

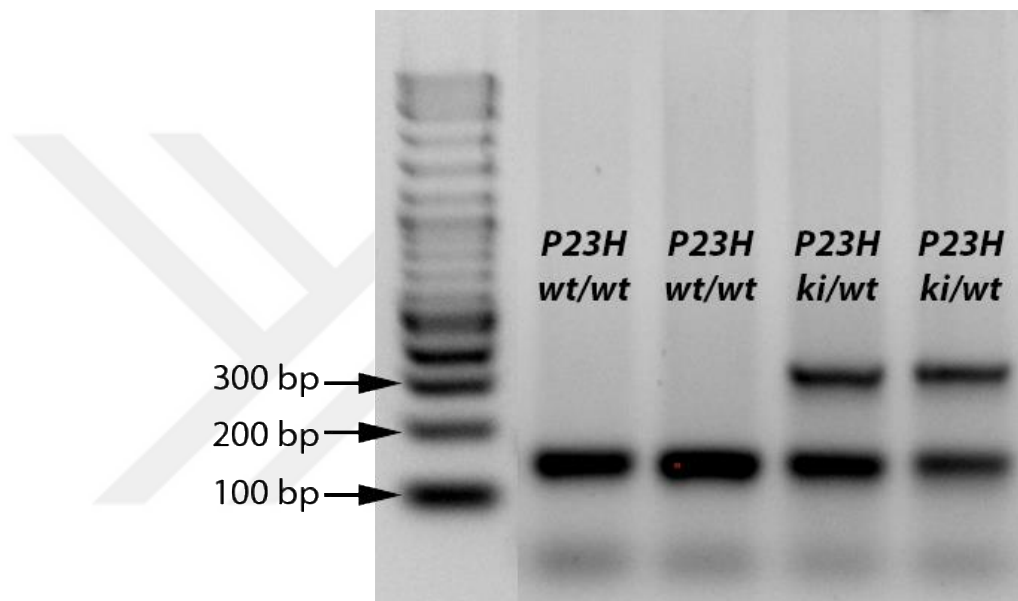


Figure 10. P23H genotyping visualized by gel electrophoresis. P23H genotyping PCR amplification results in a band at 150 bp for wild-type rhodopsin and 320 bp for P23H rhodopsin. A dual band indicates animals heterozygote for P23H mutation.

2.2.2.2. Ai14 Wt and Mutant PCR

Ai14 animals express tdTomato fluorescent protein under the CAG promoter. The vector carrying tdTomato was inserted into the *Gt(ROSA)26Sor* locus and it contains a *loxP*-flanked STOP cassette that requires Cre recombinase expression for gene expression. Ai14 genotyping PCR protocol (PCR protocol 1) was provided by The Jackson Laboratory. Two sets of primers with a common forward primer was used. Ai14 wt PCR produces a PCR band at 297 bp while the Ai14 mutant PCR produces a PCR band at 196 bp (**Fig.11**).

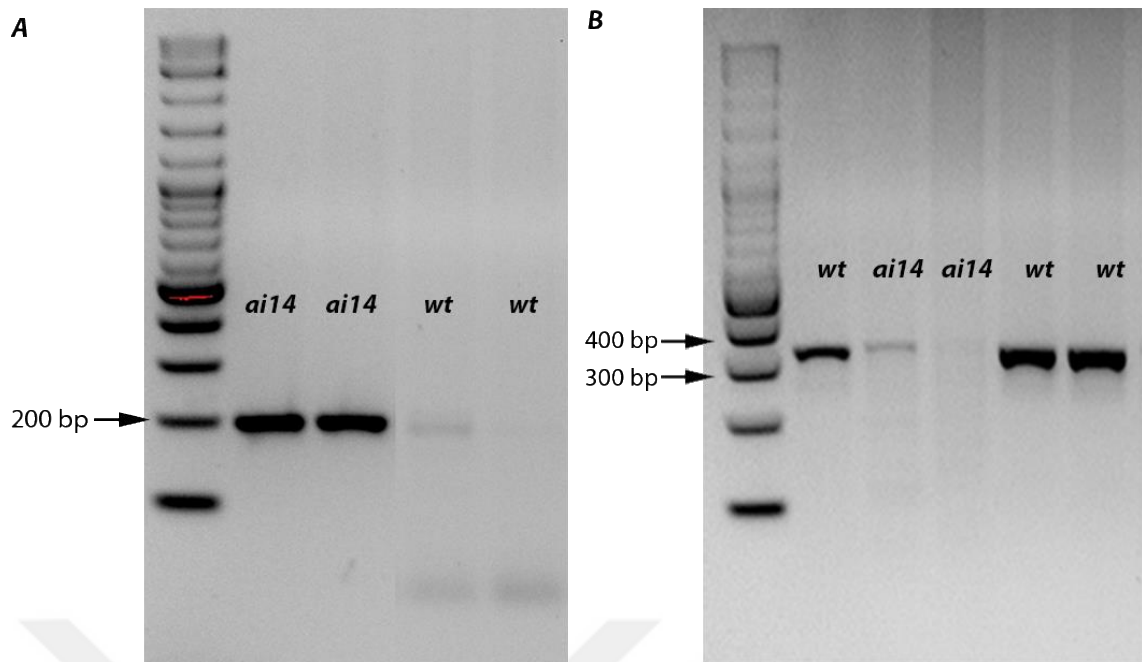


Figure 11. Ai14 genotyping PCR visualized by gel electrophoresis. Ai14 transgene can be hemizygous or homozygous. Different PCR genotypings were performed for Ai14 transgene and wild-type Rosa26. Ai14 transgenic animals show up at 196bp while wild-type animals show up at 297bp. Heterozygous animals show amplification product for both genotyping PCRs.

2.2.2.3. Rd10 PCR

Rd10 primers and PCR protocol were obtained from the originating article [50]. A DNA sample from a homozygous animal produces a band at 97 bp while a wild type animal produces a band at 54 and 43 bp. A heterozygous animal produces bands at 3 points (43, 54, 97 bp). The wild type bands are smeary however they are distinguishable (**Fig.12**).

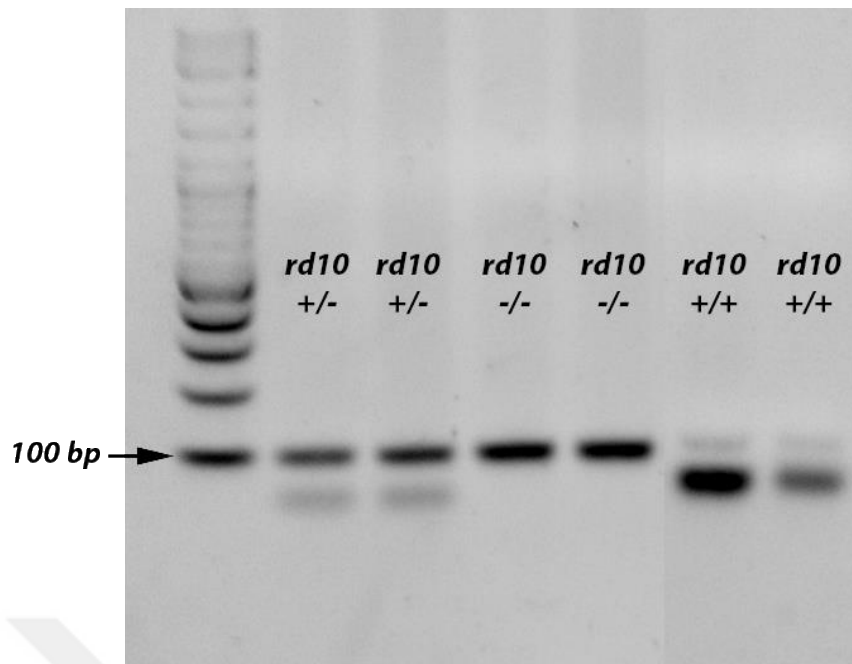


Figure 12. Rd10 genotyping PCR visualized by gel electrophoresis. Rd10 genotyping PCR products are digested by HhaI before visualization. Digested PCR products result in bands at 54bp and 43bp for wild-type Pde6b and 97bp for rd10 mutant Pde6b. Bands at 54bp and 43bp appear as a smear. As a result, Rd10 heterozygous animals show up as dual bands at 97bp and 54bp.

2.2.2.4. dCas-SPH PCR

Primers suggested by The Jackson Laboratory were used for the genotyping of dCas-SPH transgenic mice. A 264 bp PCR product was obtained using these primers. Wild-type animals tended to show up as very faint bands as seen on **figure 13**.

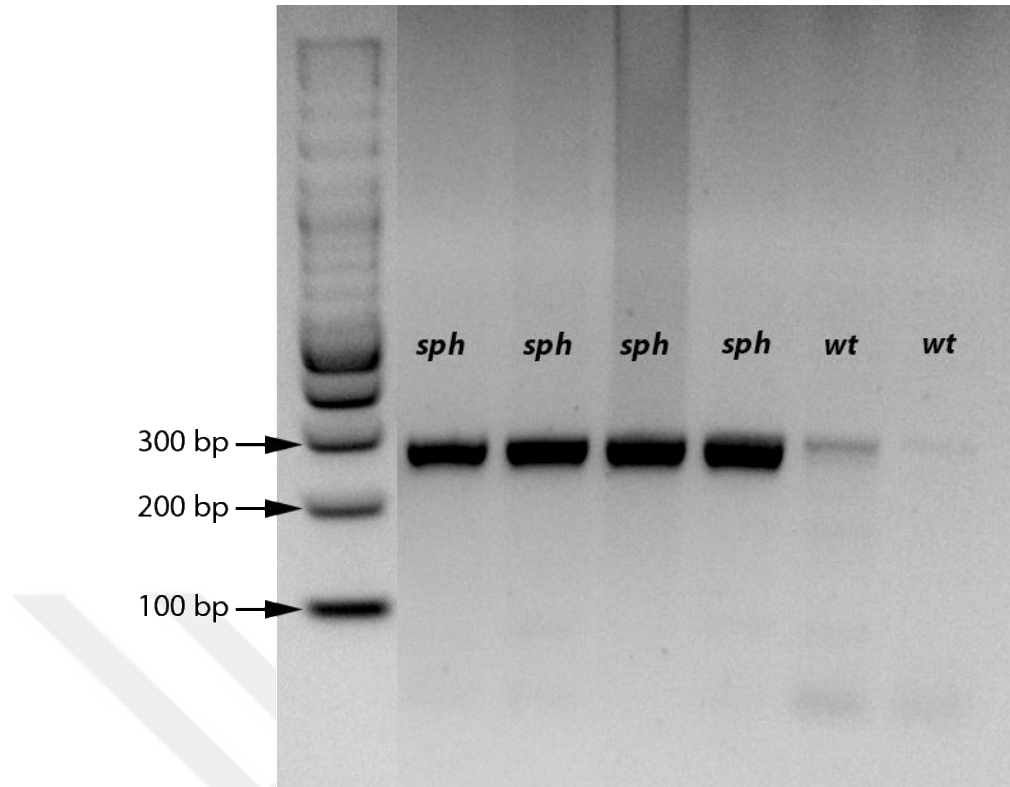


Figure 13. dCas-SPH genotyping PCR visualized by gel electrophoresis. dCas-SPH genotyping PCR results in a band at 264bp indicating transgenic animals. This PCR does not differentiate between hemizygous and homozygous animals.

2.2.3. Gel Electrophoresis

Using 242 g of Tris-base, 57.1 ml of glacial acetic acid, 100 ml of 500 mM EDTA, and ddH₂O to fill up to 1 L, a 50X TAE stock solution was created. Genotyping PCR samples were loaded onto a 2% (w/v) agarose TAE gel and run at 100V for one hour. DNA samples for gel extraction were loaded into a 0.8% (w/v) TAE agarose gel and run at 100V for an hour. DNA samples treated with restriction digestion enzyme were also loaded into a 0.8% (w/v) TAE agarose gel and run at 100V for an hour.

2.2.4. Fluorescent and Bright-Field Fundus Imaging

A 0,5% tropicamide and 2,5% phenylephrine hydrochloride eyedrop was applied to the eyes of animals to dilate the pupils. Animals were anesthetized in a chamber using %5 isoflurane delivered by an anesthesia system with a vaporizer. Isoflurane levels were reduced to 1,5% following the loss of tail-pinch response, righting reflex, and withdrawal reflex, and the animal was moved on a stage with a mouse mask for continuous isoflurane delivery. A 0,2% carbomer 980 topical gel was immediately applied to both eyes to keep them hydrated and as a coupling gel for imaging. Fluorescence and bright-field fundus imaging microscope (Phoenix, Micron IV) was used to locate and image the region with the optic nerve at its center. Ai14, dCas-SPH and Tg(Rho-Cre) transgenic animals were genotyped using retinal imaging microscope.

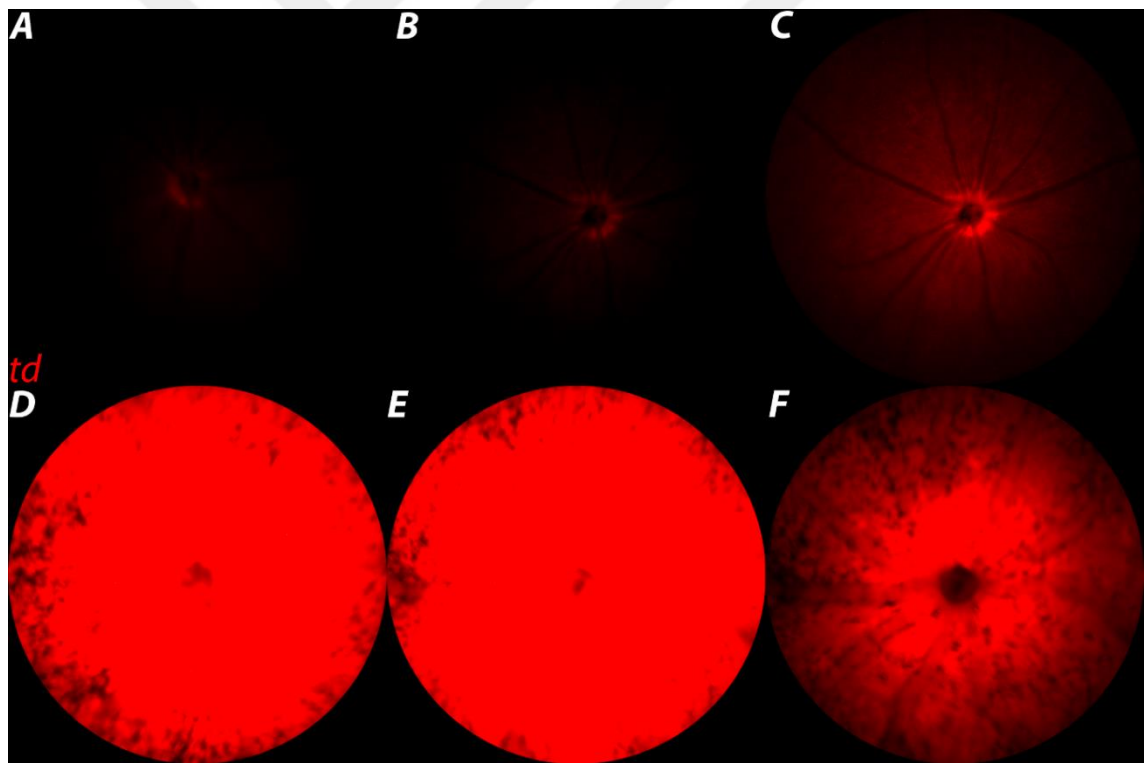


Figure 14. Ai14 leakage and Ai14-Tg(Rho-Cre) rod reporter activity visualization through retinal imaging. A, B, C) Ai14 transgenic animals show very low expression of tdTomato in fluorescent retinal imaging. C) This leaky tdTomato expression is observed better at higher exposure levels. D, E, F) Rod reporter activity in transgenic animals results in increased tdTomato expression due to Cre recombinase and Ai14 coexpression. F) A detailed pattern of rod cells can be observed at lower exposure levels. tdTomato fluorescent fundus images are taken with equal exposure settings in A, B and D, E, separately.

We observed that animals expressing Ai14 had leaky tdTomato expression by fluorescent fundus imaging (**Fig.14**). Rod reporter animals express intense amounts of tdTomato depending on the photoreceptor cell survival (**Fig.14**). Although fluorescent fundus genotyping was used initially to identify Ai14 expression, PCR genotyping was performed to confirm the transgene expression. As previously mentioned, we had problems with Tg(Rho-Cre) PCR. Thus, we only used fluorescent fundus genotyping for animals expressing Tg(Rho-Cre) transgene.

2.3. Optical Coherence Tomography (OCT)

Pupils of the animal were dilated and the animal was anesthetized using the same methods from fluorescent and bright-field fundus imaging. After moving the animal to the stage, the ophthalmic OCT device (Ocuscience, iVivo Ophthalmic OCT) was located on the area centered around the optic nerve. Focal value, power and mirror position of the device, and the position of the stage was modified for each animal to achieve the best image quality. A volume scan consisting of 512 frames was recorded for further analysis. Images recording the optic nerve (256th image $\pm$ 20 images) were selected according to image stability and the absence of artifacts as they can occur due to small movements of the animal. Selected images were loaded to the image analysis software ImageJ [121] as a stack and averaged with the Z-project tool to increase image resolution and reduce background noise. Produced images were saved in tiff file format. The depth of the OCT device was calibrated with a calibration slide provided by the manufacturer. Calibration slide contains a 190 μ m tissue and the depth per pixel of the device was configured to accommodate for the 180 μ m slip cover of the device. The width of the device was configured by opening up a caliber by 1 mm and changing the width per pixel setting until the OCT device reads 1 mm. The line tool was used for retinal thickness measurements.

2.4. Cloning

2.4.1. sgRNA Amplification

sgRNAs were amplified using a primer set that amplifies the U6 promoter region. This primer set contains MluI, XhoI and EcoRI digestion sites in the forward primer, and BglII and SpeI digestion sites in the reverse primer. The PCR reaction mixture was setup according to **table 6** and PCR protocol from **table 7** was used for amplification. Amplified sgRNA PCR products were purified using PCR purification.

Table 8. High-fidelity PCR reaction. Preparation of high-fidelity PCR reaction mixture.

High-Fidelity PCR Reaction		
Components	Volume (μl)	Final Concentration
GoTaq Green Master Mix	21	
Q5 High Fidelity DNA Polymerase	0.75	0,06 U/μl
Forward Primer	1	50 nM
Reverse Primer	1	50 nM
DNA	1-2	
Nuclease-Free Water	fill up to 25	

2.4.2. PCR Purification

PCR purification was performed using QIAquick PCR Purification Kit (QIAGEN, 28104). 5:1 (V/V) Buffer PB was added to PCR samples. 5-10 μl 3 M sodium acetate was added to samples that turned orange or violet. The mixture was added to a QIAquick spin column centrifuged for 1 min. The column was washed twice with 750 μl Buffer PE. Empty column was centrifuged for 2 mins to wash away the leftover buffers. The column was placed in a sterile 1,5 ml microcentrifuge tube and 30 μl sterile RNase/DNase-Free water was added. After waiting for 1 min, the column was centrifuged for 1 min. The DNA concentration and purity were determined using a spectrophotometer.

Table 9. High-fidelity PCR protocol. PCR protocol used for high-fidelity PCR.

High-Fidelity PCR Protocol			
1	Initial Denaturation	98°C	30 sec
2	Denaturation	98°C	10 sec
3	Annealing	66°C	30 sec
4	Synthesis	72°C	30 sec
Go back to step 2 for 34 times			
5	Elongation	72°C	2 min
6	Cool down	4°C	hold

2.4.3. Restriction Digestion

The plasmid backbone pAAV.CMV.HI.eGFP-Cre.WPRE.SV40 (pAAV-Cre, Addgene #105545) was double digested with MluI and SpeI enzymes. 0,2 µl MluI (Thermo Scientific, ER0561), 0,8 µl SpeI (Thermo Scientific, ER1251), 2 µl Buffer R (Thermo Scientific, BR5), 1 µg pAAV-Cre, and water up to 20 µl was mixed in a PCR tube. The tube was incubated at 37°C for 1 hour. The resulting products were purified using gel extraction.

Purified sgRNA was double digested using MluI and SpeI enzymes as well. 0,2 µl MluI, 0,8 µl SpeI, 2 µl Buffer R, 1 µg sgRNA DNA, and water up to 20 µl was mixed in a PCR tube. The tube was incubated at 37°C for 1 hour. The resulting products were purified using gel extraction.

Table 10. Restriction digestion. Components of restriction digestion reaction mixture and their concentrations.

Restriction Digestion Reaction		
Components	Volume (µl)	Final Concentration
Restriction Enzyme	1 units	0,1 U/µl
Restriction Enzyme Buffer	10X	1X
DNA	500 ng	30 - 100 ng/µl
DNase/RNase-Free Water	fill up to 10	

2.4.4. Gel Extraction

Gel extraction was performed to purify samples from 0.8% (w/v) TAE agarose gel using QIAquick Gel Extraction Kit (QIAGEN, 28706X4). Samples were excised from 0.8% (w/v) TAE agarose gel. 3:1 (v/v) Buffer QG was added to samples. The mixture was incubated at 50°C for 5-10 mins until the gel is dissolved. 5-10 µl 3 M sodium acetate was added to samples that turned orange or violet. The mixture was loaded to a QIAquick spin column and centrifuged for 1 mins. Then, the column was washed with 500 µl Buffer QG. The column was washed twice with 750 µl Buffer PE. Empty column was centrifuged for 2 mins to wash away the leftover buffers. The column was placed in a sterile 1.5 ml microcentrifuge tube and 30 µl sterile RNase/DNase-Free water was added. After waiting for 1 min, the column was centrifuged for 1 min. The DNA concentration and purity was determined using a spectrophotometer.

2.4.5. Ligation

T4 DNA ligase enzyme was used for ligating linearized DNA samples with compatible sticky ends. 3:1 ratio of insert to vector was used for optimal ligation. Ligation reaction

was set-up according to **table 9** and incubated at 16°C overnight. Produced ligate was stored at 4°C until further use.

Table 11. Ligation reaction. Preparation of ligation reaction mixture.

Ligation Reaction		
Components	Volume (µl)	Final Concentration
T4 DNA Ligase	0,5	6 - 20 U/µl
T4 DNA Ligase Buffer	10X	1X
Vector DNA	x	300 ng
Insert DNA	y	100 ng
DNase/RNase-Free Water	up to 10-30	

2.4.6. Competent Cell Preparation

2 µl of Stbl3 cells from the One Shot Stbl3 Chemically Competent *E. coli* kit (Invitrogen, C737303) was incubated for 12 hours in 5 ml LB. 500 µl of cultured Stbl3 cells were incubated in 50 ml of ZymoBroth at 150 rpm 30°C until the confluency reaches OD₆₀₀=0,4. After incubation, the culture was transferred to ice for 10 minutes. Cells were pelleted by centrifugation at 3000 rpm 4°C for 10 minutes. Supernatant was discarded and cells were resuspended in 5ml ice-cold 1X Wash Buffer. Cells were pelleted by centrifugation at 3000 rpm 4°C for 10 minutes and the supernatant was discarded again. Cells were resuspended in 5ml ice-cold 1X Competent Buffer. 50 µl of cells were aliquoted to microcentrifuge tubes in ethanol-dry ice bath. Aliquoted cells were stored at -80°C until further use.

2.4.7. Transformation

An aliquot of Stbl3 cells was thawed on ice. 1-5 µl plasmid DNA was added to cells on ice. Cells were incubated on ice for 30 minutes. Heat-shock was applied for 45 seconds at 42°C, and cells were incubated on ice again for 5 minutes. 1 µl of ampicillin-selective LB was added and cells were incubated at 37°C 250 rpm for 1 hour. 50-200 µl of incubated cells were spread on antibiotic-selective LB agar plates and incubated overnight at 37°C.

Table 12. Colony PCR. PCR protocol used for colony PCR.

Colony PCR Protocol			
1	Initial Denaturation	95 °C	5 min
2	Denaturation	95 °C	30 sec
3	Annealing	60 °C	30 sec
4	Synthesis	72 °C	1 min
	Go back to step 2 for 34 times		
5	Elongation	72°C	10 min
6	Cool down	4°C	hold

2.4.8. Colony PCR

Colonies were selected from agar plates using pipette tips and grown overnight in 3 ml ampicillin (100 µg/ml) selective LB media at 37°C 250 rpm. 1 µl of bacterial culture was directly used for colony PCR. The remaining bacterial cultures were stored at 4 °C until further use. Colony PCR protocol was created by modifying the annealing temperature of PCR protocol 3 to 60 °C. PCR products were loaded on a 2% agarose gel and run at 100V for 1 hour.

2.4.9. Small-Scale Plasmid Isolation

A commercial kit (Zyppy Plasmid Miniprep Kit, D4019, Zymo Research) was used for the small-scale isolation of plasmid DNA. 2,9 ml of the bacterial culture remaining from the colony PCR was used for small-scale plasmid DNA isolation. 2,9 ml bacterial culture was transferred to microcentrifuge tubes and centrifuged for 30 seconds at maximum speed. The supernatant was discarded and 600 µl of DNase/Rnase-Free sterile water was used to resuspend the cell pellet. 7X Lysis Buffer was added and the tube was mixed by inverting several times. The cell pellet was lysed for 2 minutes and 350 µl of ice cold Neutralization Buffer was added immediately. The tube was inverted several times and centrifuged at 16000g for 4 minutes. The supernatant was poured over into the Zymo-Spin IIN column that is placed in a Collection Tube. The tube was centrifuged at max speed for 15 seconds and the Collection Tube was emptied. The column was washed by adding 200 µl Endo-Wash Buffer and centrifuging for 30 seconds at max speed. Then, the column was washed with 400 µl Zyppy Wash buffer and centrifuged for 1 minute. The column was transferred to a clean microcentrifuge tube and 30 µl DNase/Rnase-Free sterile water was added on the membrane. The tube was incubated at room temperature for 1 minute and centrifuged for 30 seconds at max speed. A UV-Vis spectrophotometer was used to measure the concentration of plasmid DNA.

2.4.10. ITR Stability Check

As AAV ITRs contain XmaI digestion sites, AAV plasmid vectors were digested with XmaI (R0180L, New England Biolabs) according to **table 8**. Digestion products were loaded in a 0,8% agarose gel and run at 100V for 1 hour (**Fig.15**). Vectors with stable ITR sequences were sent to Sanger sequencing.

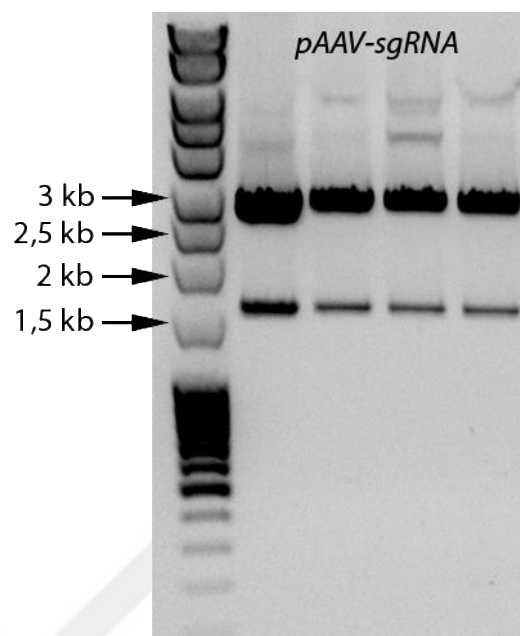


Figure 15. ITR stability testing of pAAV-sgRNA vectors. pAAV-Cre vectors with sgRNA insert (pAAV-sgRNA) contains AAV2 ITR sequences at 5' and 3' ends. Each ITR contains 2 XmaI digestion sites and another XmaI digestion site is found before eGFP. XmaI digestion reaction produces DNA fragments with bands 3137 bp, 2718 bp, and 1491 bp. 3137 bp and 2718 bp fragments appear as a smear.

2.4.11. Sanger Sequencing

Cloned plasmids were sent to a specialized company (Medsantek) to perform Sanger sequencing. 20 µl of 100 ng/µl plasmids were sent together with 10 µl of 10 µM primers. Two-way sequencing was performed for each plasmid. Sequencing results were analyzed using Qiagen CLC Main Workbench.

2.4.12. Large-Scale Plasmid Isolation

For large-scale plasmid isolation, a commercial kit devoid of endotoxins (NucleoBond Xtra Midi EF, 740420.50, Macherey-Nagel) was employed. The remaining 100 µl of bacterial culture was transferred to a 1000 ml flask containing 100 ml of ampicillin-

selective LB media and it was incubated overnight at 37 °C 200 rpm. Then, the bacterial culture was centrifuged for 15 minutes at 6000 g and 4 °C. The pellet was resuspended in 8ml resuspension buffer after the supernatant was removed. The mixture received 8 ml of lysis buffer and was left to sit at room temperature for 5 minutes. After adding 8ml of neutralization buffer to the lysate, the mixture was allowed to sit at 4 °C for five minutes. A column was pre-wetted with 15 ml of equilibration buffer. The lysate was emptied into the pre-wetted column. Using 5 ml of the FIL-EF buffer, the column and filter were washed. The column was washed with 35 ml of buffer ENDO-EF after the filter was discarded. Column was cleaned then with the buffer WASH-EF. Using 5 ml of the buffer ELU-EF, the plasmid DNA was eluted. Plasmid DNA was then precipitated by adding 3.5 ml of room temperature isopropanol to the elute after elution. The elute was centrifuged for 30 minutes at 15000 x g, 4 °C. 2 ml Room-temperature 70% endotoxin-free ethanol was added to the pellet after the supernatant was removed and the tube was centrifuged at 15000 x g, 22 °C for 5 minutes. The pellet was dried for 15 minutes after the ethanol was removed. A UV-Vis spectrophotometer was used to measure the concentration of plasmid DNA after the pellet was resuspended in 200 µl of H₂O-EF.

2.5. Large-Scale AAV Production

2.5.1. Cell Culture

HEK293T cells were previously bought and cultured in the lab. Cells were kept in cell culture incubators at 37°C and supplied with 5% CO₂.

2.5.1.1. Cell Thawing

Frozen vials were thawed in a 37°C water bath. Thawed cells were resuspended in 1 mL of Dulbecco's Modified Eagle Medium (High Glucose DMEM, D5796, Sigma-Aldrich) containing penicillin-streptomycin (P/S, P06-07100, PAN Biotech) (100 U/ml Penicillin, 100 µg/ml Streptomycin) and 10% fetal bovine serum (FBS, 10500056, Gibco). Resuspended cells were transferred to 150 mm cell culture plates (93150, TPP) containing 21 mL DMEM (w/P-S, w/2% FBS). The plate was shaken gently and placed in a cell culture incubator.

2.5.1.2. Cell Passaging

HEK293T cells were maintained in 150 mm cell culture dishes containing DMEM media with 10% FBS and 1% Pen-Strep. Media was changed once every two days if deemed necessary. Cells were passaged once after thawing, before starting an experimental setup. Cells at 80% confluency were passaged into three cell culture dishes. These cells were passaged into 10 150 mm cell culture dishes when they reach 80% confluency.

2.5.1.3. Cell Freezing

A freezing media containing 1 ml DMSO and 9 ml DMEM (w/PS, w/FBS) was prepared. Plate was washed with PBS (P04-36500, PAN Biotech) once and trypsinized for 2 minutes. 9 ml DMEM (w/PS, w/FBS) was added to the plate and cells were collected in a 15 ml Falcon tube. Cells were centrifuged at 200 rpm for 5 minutes. Supernatant was removed and freezing media of necessary amount was added to resuspend the cell pellet.

Cells were transferred to cell freezing vials and frozen to -80°C gradually using freezing containers (Mr.Frosty Freezing Container, 5100-0001, Thermo Scientific).

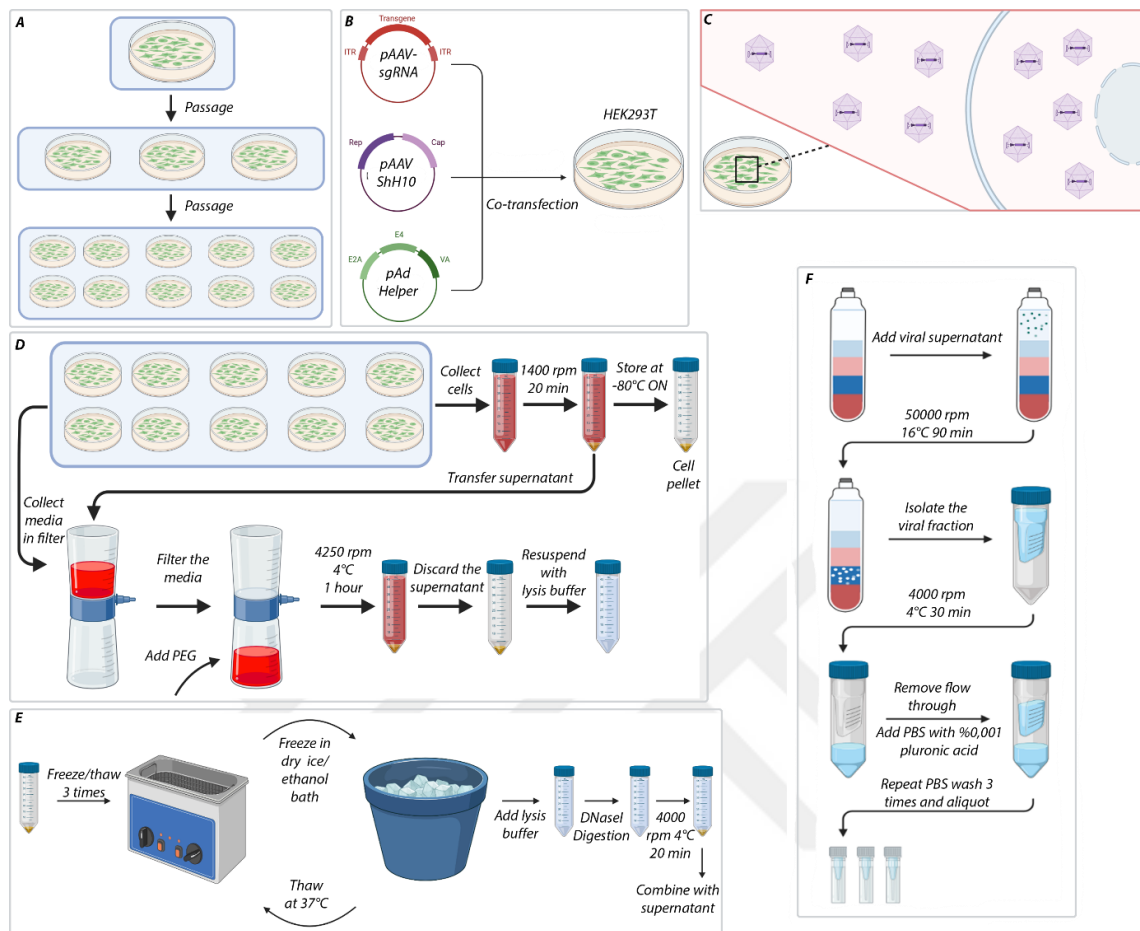


Figure 16. Large-scale AAV production. **A)** HEK cells are passaged into 3 cell culture dishes and then into 10 cell culture dishes. **B)** Cells are PEI cotransfected with pAAV-sgRNA, helper plasmid and serotype plasmid. **C)** AAV particles produced by the cell are concentrated in the cell and secreted to the media as well. **D)** 2 days later, 16ml media from each plate was 0,22 μm filtered. The remaining media on the plate is used to detach the cells from the cell culture dish. Cells are collected in a centrifuge tube and centrifuged at 1400 rpm for 20 min. The supernatant was 0,22 μm filtered together with the media. The cell pellet was stored overnight at -80°C . PEG was added to the filtered media, and the mixture was collected in a centrifuge tube and stored overnight at 4°C . The next day, media with PEG was centrifuged at 4250 rpm 4°C for 1 hour. The supernatant was discarded and the pellet was resuspended with 18 ml of lysis buffer. **E)** The cell pellet was freeze-thawed three times between a 37°C water bath and a dry ice/ethanol bath. The cell pellet was resuspended with 15 ml lysis buffer and digested with turbonuclease. Digested cell pellet was centrifuged at 4000 rpm 4°C for 20 min and the supernatant was added to the filtered centrifuged media. **F)** The collected viral media was added onto the iodixanol gradient and topped off with PBS. The gradient was centrifuged at 50000 rpm 16°C for 90 min. The viral fraction was isolated and PBS containing 0,001% Pluronic F-68 up to 15 ml was added to the fraction. The viral fraction was filtered through a cellulose filter unit. Then, the same filter unit was washed with PBS containing 0,001% Pluronic F-68 three times. The purified AAV fraction remaining in the filter unit was aliquoted and stored at -80°C . Figure created with BioRender.com.

2.5.2. Transfection

2.5.2.1. Polyethylenimine (PEI) Preparation

Transfection grade linear PEI (PEI MAX, 24765-1, Polysciences Inc.) with 40000 molecular weight was dissolved in DNase/RNase-free sterile water to a final concentration of 1 mg/ml. The mixture was stirred at 30 to 50 °C for 1 hour to completely dissolve the powder. The solution's pH was adjusted to 7 and it was filter-sterilized through a 0,22 µm vacuum filter. Aliquots were made and stored at -20°C.

2.5.2.2. PEI Transfection

A transfection mixture containing 70 µg AAV plasmid, 70 µg AAV serotype plasmid (pAAV2/ShH10, pAAV-BP2), 200 µg adenoviral helper plasmid (pHGTI-aden01), 49 ml DMEM, and 1360 µl of PEI was prepared in a 50 ml centrifuge tube. The tube was inverted gently and incubated at room temperature for 15 minutes. The transfection mixture was distributed 5 ml each to 10 150 mm cell culture dishes.

2.5.3. AAV Harvest

2.5.3.1. Poly(ethylene glycol) (PEG) Preparation

200 g of PEG (P5413, Sigma-Aldrich) was added to 250 ml 5M NaCl. DNase/RNase-free sterile water was added to the mixture to bring the volume up to 500 ml. The mixture was kept at 37°C overnight and filtered through a vacuum filter (Stericup Quick Release, S2GPU05RE, Millipore)

2.5.3.2. From Supernatant

48 to 60 hours after transfection, 16 ml of supernatant from each 150 mm culture dish was collected and filtered in a 0,22 µm 250 ml vacuum filter (Stericup Quick Release, S2GVU02RE, Millipore). The cells remaining on the dish were thoroughly resuspended and collected into 50 ml centrifuge tubes. Resuspended cells were centrifuged at 1400 rpm for 20 minutes. The supernatant was filtered through the same vacuum filter. The cell pellet was stored at -80°C overnight. 50 ml sterile filtered PEG was added to the filtered supernatant and the mixture was stored at 4°C overnight. The next day, supernatant mixed with PEG was centrifuged at 4250 rpm 4°C for 1 hour. The PEG pellet was resuspended in 18 ml of filtered lysis buffer containing 150 mM NaCl and 20 mM tris pH 8.

2.5.3.3. From Cells

The cell pellet was freeze-thawed three times using dry ice-ethanol bath and 37°C water bath. Then, the cell pellet was resuspended using 15 ml lysis buffer. Resuspended cell pellet was digested with 15 µl of nuclease (TurboNuclease, N0103L, Accelagen Inc.), supplemented with 15 µl 1M MgCl₂, at 37°C for 30 minutes. Following digestion, cell debris was centrifuged at 4000 rpm 4°C for 20 minutes. Isolate the supernatant and add it to the resuspended PEG pellet.

2.5.4. Iodixanol Solution Preparation

4 solutions containing different percentages of iodixanol in the following order were prepared: 17%, 25%, 40%, 60%. For 17% iodixanol solution, 20 mL 10X PBS, 200 µl 1M MgCl₂, 500 µl 1M KCl, and 40 ml 5M NaCl was mixed with 50 ml iodixanol (OptiPrep Density Gradient Medium, D1556, Sigma-Aldrich). Water was added up to 200 ml. For 25% iodixanol solution, 20 mL 10X PBS, 200 µl 1M MgCl₂, 500 µl 1M KCl, and 800 µl 0,5% phenol red (P0290, Sigma-Aldrich) was mixed with 80 ml iodixanol. Water was added up to 200 ml. For 40% iodixanol solution, 20 mL 10X PBS, 200 µl 1M MgCl₂, and 500 µl 1M KCl was mixed with 133,2 ml iodixanol. Water was added up to 200 ml. For 60% iodixanol solution, 200 µl 1M MgCl₂, 500 µl 1M KCl, and 200 µl 0,5% phenol red was mixed with 200 ml iodixanol.

2.5.5. Iodixanol Gradient Preparation

Iodixanol gradient is produced by very slowly pipetting 4 ml of 60%, 40%, 25% and 17% solutions respectively into a polypropylene tube (OptiSeal Polypropylene Tube, 362183, Beckman). Supernatant with the AAV was added into the propylene tube. Any empty space was filled by adding PBS. The polypropylene tube was sealed with a plug (Large Plug, 361658, Beckman). The tube was carefully placed in the vertical angle rotor (VTi 50, Beckman) and sealed with a spacer (OptiSeal Spacer, 362204, Beckman). The polypropylene tube was centrifuged at 50000 rpm 16°C for 90 minutes. The viral fraction was isolated from the 40% layer using a 21G needle. The needle was inserted from approximately 2 mm below the intersection of 60% and 40% layers. 3 ml of 40% layer was carefully extracted without any of the 25% layer.

2.5.6. AAV Fraction Purification

A 15 ml cellulose filter unit (Amicon Ultra-15 Centrifugal Filter Unit, UFC910008, Millipore) was pre-wetted with PBS. The extracted virus fraction was transferred to a 50 ml centrifuge tube and PBS containing 0,001% Pluronic F-68 (Non-ionic Surfactant, 24040032, Gibco) was added up to 15 ml. The mixture was added to the filter unit and centrifuged at 4000 rpm 4°C for 30 minutes. The flow-through was discarded and PBS containing 0,001% Pluronic F-68 up to 15 ml was added to the filter unit. The filter unit was gently inverted several times and centrifuged at 4000 rpm 4°C for 30 minutes. These PBS containing 0,001% Pluronic F-68 wash and centrifuge steps were repeated 2 more times in the same filter unit. After emptying the flow-through one last time, the remaining virus fraction on the filter unit was aliquoted 20 µl each into screw cap tubes on ice. One aliquot containing 5 µl of AAV was kept at 4 °C for titration and the rest of the aliquots were stored at -80 °C.

2.5.7. DNase Treatment

5 µl of purified AAV was digested with 5 µl DNaseI (M0303S, New England Biolabs) supplemented by 5 µl 10X reaction buffer and water up to 50 µl. The reaction mixture was incubated at 37°C for 30 minutes and then 95°C for 10 minutes.

2.5.8. Digital Droplet PCR (ddPCR) Titration

10-fold serial dilution was performed 4 times with the digested AAV products. 10-fold serial dilutions were performed using 0,05% Pluronic F-68 solution in a LoBind 96-well PCR plate (twin.tec PCR Plate 96 LoBind, 0030129512, Eppendorf). Primers binding to the woodchuck hepatitis post-transcriptional regulatory element (WPRE) were used for titration. A ddPCR reaction mastermix without the DNA sample was prepared according to **table 11**. ddPCR reaction mastermix was distributed to a LoBind 96-well PCR plate. 3rd and 4th AAV dilutions were used for ddPCR reaction. The DNA samples were distributed carefully to each well. 20 µl ddPCR reaction mixture from each well were transferred to a ddPCR cartridge (DG8 Cartridge, 1864008, Biorad). 70 µl droplet generation oil for probes (1863028, Biorad) or EvaGreen (1864006, Biorad) was aspirated to the cartridge. The cartridge was sealed with a gasket (DG8 Gaskets, 1863009, Biorad) and loaded on a droplet generator (QX200 Droplet Generator, 1864002, Biorad). Following droplet generation, droplets were transferred to a 96-well ddPCR plate (12001925, Biorad). The ddPCR plate was heat sealed with an aluminum sealing foil (PCR Plate Heat Seal, 1814040, Biorad). The ddPCR plate was placed in a thermal cycler and the PCR protocol from **table 12** was used. Lastly, the ddPCR plate was placed on the droplet reader (QX200 Droplet Reader, 1864003, Biorad) for fluorescent signal detection and analysis.

Table 13. Standard ddPCR Reaction. Preparation of the standard ddPCR reaction mixture.

Standard ddPCR Reaction	
Components	Volume (µL)
Reaction Supermix	11
Primer or Probe Mix	1
DNA	1
Water	9

Table 14. ddPCR Protocol. PCR protocol for amplification of ddPCR products.

ddPCR Protocol		
1	95°C ramp 2°C/sec	5 min
2	95°C ramp 2°C/sec	30 sec
3	60°C ramp 2°C/sec	1 min
Go back to step 2 for 39 times		
4	4°C ramp 2°C/sec	5 min
5	90°C ramp 2°C/sec	5 min
6	4°C ramp 2°C/sec	hold

2.6. Intravitreal Injection

A 10 µl microsyringe (Sub-Microliter Injection System, NANOFIL, World Precision Instruments) with a 36G blunt-end needle was washed 5 times with 70% EtOH, DNase-Rnase-free sterile water, and PBS. The necessary amount of AAV was aspirated into the syringe. Prior to injection, all animals were imaged using optical coherence tomography and fluorescent and bright-field fundus imaging. After imaging, a 0,3% tobramycin and 0,1% dexamethasone anti-inflammatory and anti-bacterial eye drop was applied to both eyes. A surgical suture was used to pierce through the eyelid and was taped down to stabilize the position of the eyelid. A small portion of the conjunctiva around the injection area was removed gently using curved iris scissors. The exposed sclera was punctured using a sterile 30G insulin syringe. The microsyringe was placed on a micromanipulator and guided through the punctured hole into the vitreous. The appropriate amount of AAV was slowly dispensed into the vitreous. Needle was slowly removed after 1 minute. The suture was removed and a 0,3% tobramycin and 0,1% dexamethasone anti-inflammatory and anti-bacterial topical gel was applied to each eye. The animal was monitored until recovery and placed in its cage.

2.7. Fluorescent Activated Cell Sorting

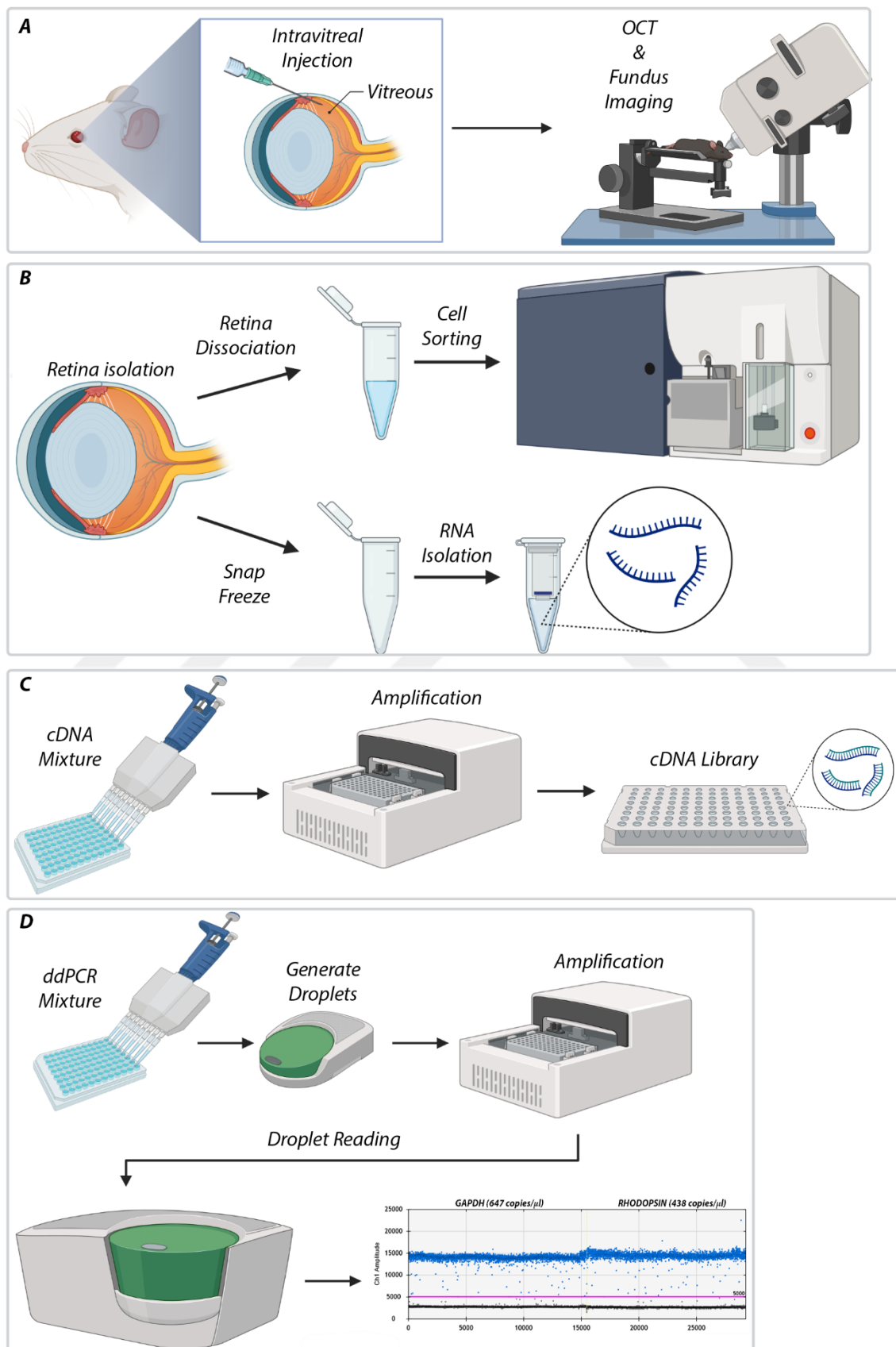


Figure 17. Intravitreal AAV-sgRNA injection readout. **A)** Mice were intravitreally injected with AAV-sgRNA and the eyes were imaged biweekly after injection. **B)** Retinas were isolated and isolates were either dissociated and cell sorted or snap-frozen in liquid nitrogen and RNA isolated. **C)** cDNA libraries were produced by a reverse transcription reaction either directly from sorted cells or from RNA samples. **D)** cDNA libraries were analyzed by ddPCR amplification. Specific gene targets were amplified and their expression levels were normalized to housekeeping genes (such as Gapdh). Figure created with BioRender.com.

2.7.1. Whole Retina Isolation and Dissociation

A variety of solutions and materials were prepared prior to whole retina isolation and dissociation. 1M HEPES was diluted in HBSS to a concentration of 100mM. A 25 mM l-cysteine solution was prepared by dissolving 30,3 mg l-cysteine in 10 ml DNase/RNase-free sterile water. 25 mM l-cysteine was aliquoted 100 µl each into microcentrifuge tubes and stored at -20°C until further use. 5 mM EDTA solution was prepared by dissolving 73 mg of EDTA in 50 ml DNase/RNase-free sterile water. A dissociation mixture containing 320 µl 100 mM HEPES, 40 µl papain (10108014001, Roche), 20 µl 25 mM l-cysteine, and 20 µl 5 mM EDTA was prepared fresh for each retina to be dissociated. Dissociation mixture was incubated at 37°C for 15 minutes and was distributed into microcentrifuge tubes if prepared in bulk. 3 ml of colorless DMEM (31053028, Gibco) mixture containing 2% FBS, 0,025% kolliphor-P188, and 0,1% Draq7 (424001, Biolegend) was prepared for each retina sample. 3ml of colorless DMEM mixture without Draq7 was prepared for control samples as well. Tips of glass pPasteur pipettes (3233050, Marienfeld) were polished by heat.

Mice were sacrificed by CO₂ and their corneas were sliced open using a scalpel. Lens and vitreous were removed by squeezing the eyecup with forceps. Using forceps, whole retinas were isolated and transferred to a 1,5 ml microcentrifuge tube containing dissociation mixture. Tubes were incubated at 37°C for 7 minutes. The dissociation mixture was discarded and 1 ml of colorless DMEM mixture was added. The whole retinas were dissociated by passing the mixture up and down through the glass pasteur pipettes using a rubber suction ball. Dissociated retinas were filtered through a 70 µm cell strainer (pluriStrainer, 431007060, Pluriselect) into a 5 ml round bottom tube (352235,

Falcon). 2 ml of colorless DMEM mixture was added through the 70 µm cell strainer into the tube. The tube was placed on ice.

2.7.2. Cell Sorting

Cells either grown *in vitro* or isolated from retina were sorted using a BD FACSAria Fusion flow cytometer device in the cell sorting facility of TUBITAK MAM. BD FACSDiva software was used for cell selection through various channels. Cells were selected through forward scatter/side scatter, Apc-cy-7, and FITC channels. Apc-cy-7 channel reports the Draq7 fluorescence, which is a cell death marker. 600 cells were sorted into a microcentrifuge tube containing 3 µl TM buffer. TM buffer was prepared by diluting the stock solution 1:10 in ddH₂O. Then, 1 U/µl of RNase inhibitor was added to the dilution. Sorted cells were immediately placed on dry ice. Sorted cells were stored at -80°C until further use.

2.8. Whole Retina Snap-Freeze

Mice were sacrificed by CO₂ and their corneas were sliced open using a scalpel. Lens and vitreous were removed by squeezing the eyecup with forceps. Whole retinas were isolated and transferred to a 1,5 ml microcentrifuge tube using forceps. Tubes containing whole retinas were snap-frozen in liquid nitrogen. Snap-frozen whole retinas were stored at -80°C until further use.

2.9. cDNA Library

2.9.1. From Sorted Cells

A cDNA reaction mixture was prepared according to **table 13** in a LoBind 96-well PCR plate. The reaction mixture was stirred vigorously after adding RNA samples. The plate was incubated in a thermal cycler following the cDNA protocol **table 15**.

Table 15. Reverse transcription reaction for sorted cells. Preparation of the reverse transcription reaction mixture for sorted cells.

cDNA Mix - Cells		
Components	Volume (µL)	Concentration
Reverse Transcriptase	0,450	50 U/µl
RNase Inhibitor	0,225	
Reaction Buffer	0,9	10X
dNTP	0,360	100 mM
Random Primer	0,9	10X
RNA	1	
water	5,165	

2.9.2. From Whole Retina

2.9.2.1. RNA Extraction

RNA extraction was performed using the High Pure RNA Isolation Kit (Roche, 11828665001). 400 µl lysis buffer was added to snap frozen retinas which were then homogenized using a 21 G needle. 190 µl of absolute ethanol was added to homogenized retinas and mixed thoroughly. The resulting lysate was transferred to Roche Spin Column and centrifuged for 1 minute at 16000 g. DNase-Mix containing 20 µl DNase and 80 µl

DNase Incubation Buffer was prepared beforehand. The flow-through was discarded and 100 µl of DNase-Mix was added to the membrane. The membrane with DNase-mix was incubated at room temperature for 15 minutes. Following incubation, 500 µl of Wash Buffer I was added to the column and centrifuged for 1 minute at 16000 g. Flow-through was discarded. 500 µl of Wash Buffer II was added to the column. The column was centrifuged for 1 minute at 16000 g and low-through was discarded. 200 µl of Wash Buffer II was added to the column and centrifuged for 1 minute at 16000 g. Flow through was discarded and the empty column was centrifuged for 2 minutes at 16000 g. The membrane was transferred to a clean 1,5 ml microcentrifuge tube and 50 µl of DNase/RNase-free water was added to the membrane. RNA concentration was determined using a UV-Vis spectrophotometer.

2.9.2.2. cDNA Production

A cDNA reaction mixture was prepared according to **table 14** in a LoBind 96-well PCR plate. The reaction mixture was stirred vigorously after adding RNA samples. The plate was incubated in a thermal cycler following the cDNA protocol from **table 15**.

Table 16. Reverse transcription reaction for whole retina. Preparation of the reverse transcription reaction mixture for whole retina.

cDNA Mix - Whole Retina		
Components	Volume (µL)	Concentration
Reverse Transcriptase	1.25	50 U/µl
Reaction Buffer	5	10X
dNTP	2	100 mM
Random Primer	5	10X
RNA	x	
water	50 - (x + 13,25)	

Table 17. Reverse transcription protocol. Protocol for the reverse transcription of cDNA.

Reverse Transcription Protocol		
1	25°C	10 min
2	37°C	90 min
3	85°C	5 min
4	4°C	hold

2.10. ddPCR

Primers for FGF2, CNTF, MANF, LIF to be used for ddPCR were designed using Primer-Basic Local Alignment Search Tool (Primer-BLAST, NCBI, <https://www.ncbi.nlm.nih.gov/tools/primer-blast>). The protocol from section 3.5.8 was followed for ddPCR analysis of gene expression.

2.11. Statistical Analysis

All experimental animal groups were selected to contain age-matched animals with the same genetic background. Results from in vivo experiments were statistically analysed for significance. Multiple timepoint long-term follow-ups were analysed through multiple unpaired Welch t-test. Multiple cohort sample groups were analyzed through multiple unpaired Welch t-test as well. Single cohort experiment retinal thickness measurement results were analyzed in a paired manner comparing treated eye to the control eye through ratio-paired t-test. All statistical analysis were performed using GraphPad Prism version 9.1.2 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com.

3. RESULTS

3.1. Generation of a P23H Rod Reporter Mouse Model

Rhodopsin mutations are a major cause of IRDs and P23H is the most prevalent mutation in adRP patients as mentioned previously. This made P23H a major focus point in animal model research. P23H mutations were introduced to *Caenorhabditis elegans* [122], *Danio rerio* [123], *Mus musculus* [48], and *Sus scrofa domesticus* [124]. We decided to use P23H mouse model due to ease of maintenance of mouse compared to other larger species. P23H mouse model was already characterized using methods such as OCT, electroretinography, immunohistochemistry and electron microscopy [48]. We wanted to utilize a different approach in which the survival of photoreceptors could be tracked over time in a retinal degeneration mouse model. Thus, we generated a P23H reporter mouse line that expresses tdTomato specifically in rod photoreceptor cells. This was achieved by crossing Ai14 animals that express tdTomato in the presence of Cre recombinase, and Tg(Rho-Cre) animals that express Cre recombinase under the rhodopsin promoter. Rod reporter animals express tdTomato only in rod photoreceptors and they make it possible to track rod photoreceptor cells noninvasively. Rod reporter animals were crossed with P23H animals to obtain the triple transgenic P23H rod reporter mouse model. P23H rod reporter mice were kept P23H heterozygous, as these animals show a slower rate of degeneration [48].

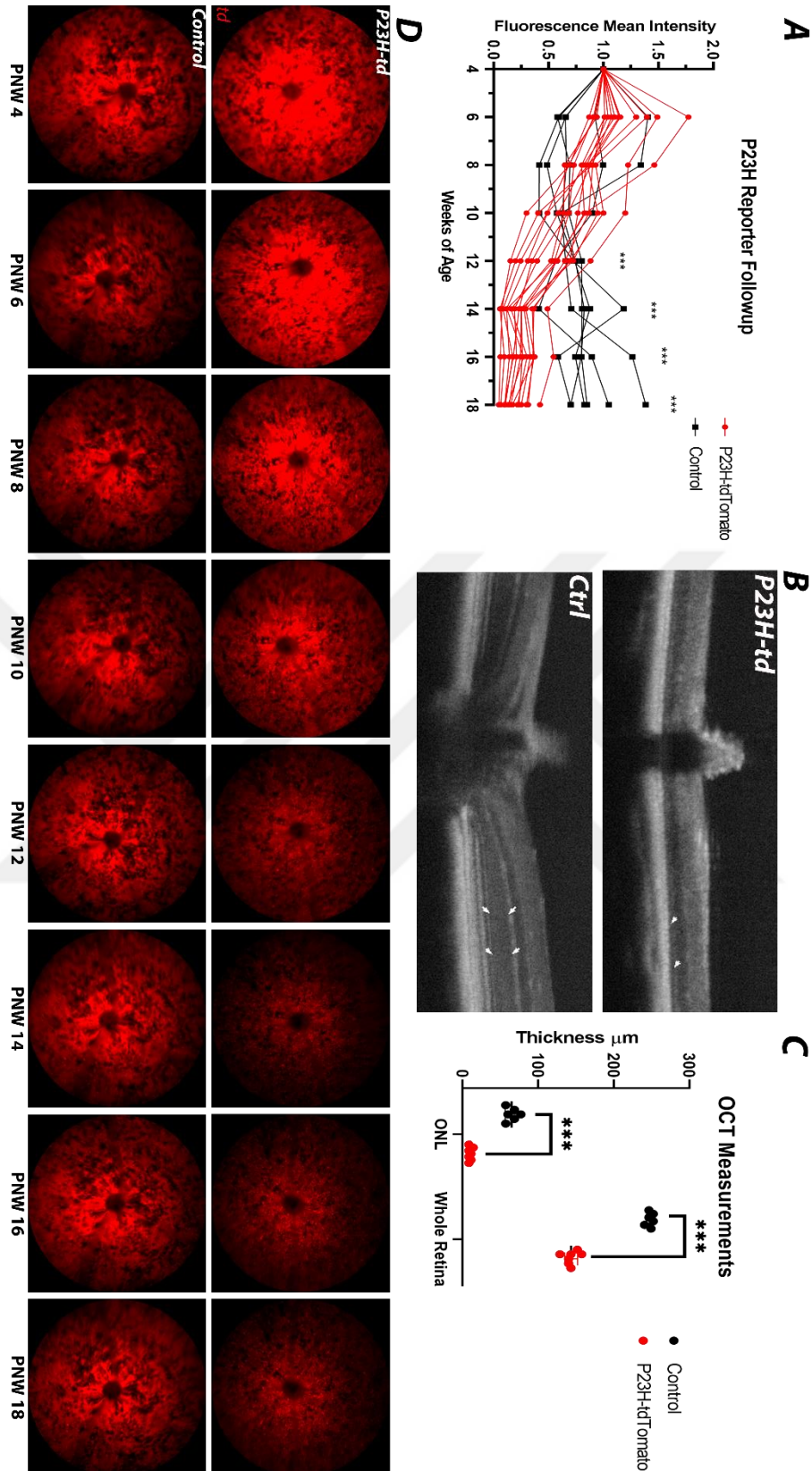


Figure 18. P23H rod reporter mouse model follow-up study. P23H heterozygous ($n=15$) and P23H wild-type rod reporter ($n=6$) mice retinas were fluorescent and bright-field fundus imaged biweekly from PNW 4 to PNW 18. **A)** The fluorescence intensity of fluorescent fundus images were quantified for each week. **B)** Retinas were OCT imaged at PNW 18 and **C)** ONL and whole retina thickness measurements were performed. **D)**

*P23H heterozygous retina experiences gradual rod photoreceptor loss indicated by the decrease in fluorescence intensity. Rod cell loss is reflected in retinal thickness measurements from OCT images as well. Multiple unpaired Welch t-tests were performed for statistical analysis in A, C. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for tdTomato. *** indicates $p < 0,0015$.*

3.1.1. P23H Rod Reporter Mouse Line Follow-up

Both eyes of 4 weeks old P23H reporter mice were bright-field and fluorescent fundus imaged. Then, these animals were fundus bright-field and fluorescence imaged biweekly until they reached 18 weeks. At 18 weeks, animals were imaged with OCT as well. Fluorescence brightness levels were measured using ImageJ software for each time point. P23H reporter retinas showed a gradual decrease in fluorescence intensity while control retinas showed a steady fluorescence intensity throughout the study (**Fig.18 D**). It was observed that a significant photoreceptor degeneration is reflected by the reduced fluorescent intensity by PNW 12. Almost all of the photoreceptor cells are degenerated by week 14, as the fluorescent levels are significantly reduced at each time point after PNW 14 compared to control retinas (**Fig.18 A**). OCT imaging revealed that P23H reporter retinas had much thinner outer nuclear layer (ONL) compared to the control group at PNW 18 (**Fig.18 B, C**). We concluded that week 4-6 was the ideal timepoint for a neuroprotective treatment as it is before the start of degeneration and the effects of treatment should delay the degeneration of ONL.

3.2. pAAV-sgRNA Vector Cloning

We previously produced a set of sgRNAs targeting *mus musculus Cntf*, *Manf*, *Lif*, *Fgf2* genes and cloned them upstream of a gRNA scaffold in a GFP reporter vector (PCA01, **Fig.19**). An *in vitro* screen was performed using a dCas9-VP64 system *in vitro* in our lab before. This was done by delivering sgRNA vectors through transfection into dCas9-

VP64 stable Müller glia cells that were produced in our lab as well. Based on our previous unpublished data, we selected sgRNAs with the highest overexpression levels. These sgRNAs were cloned into an AAV vector using a cloning strategy that makes it possible to multiplex sgRNAs in a single AAV vector.

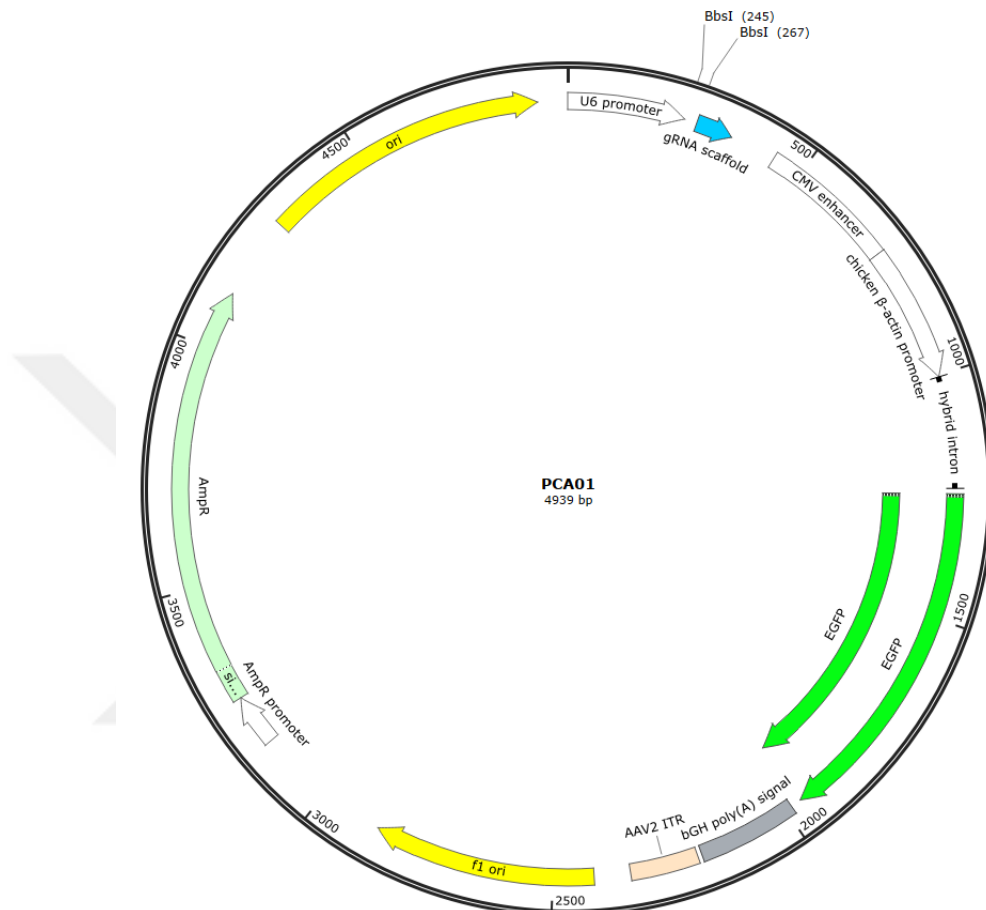


Figure 19. A map of the PCA01 plasmid. PCA01 plasmid contains U6 promoter for efficient expression of the sgRNA. The vector expression is controlled by chicken β actin promoter (CBA) and cytomegalovirus (CMV) enhancer. Vector expression can be tracked by eGFP expression.

3.2.1. pAAV-sgRNA Vector Design

An AAV vector called pAAV-Cre (pAAV.CMV.HI.eGFP-Cre.WPRE.SV40, Addgene #105545) was used as the backbone. Primers with restriction enzyme insertions were designed to clone the 423 bp region containing the U6 promoter, sgRNAs, and the gRNA

scaffold into this vector. Forward primers target upstream of U6 promoter while reverse primers target downstream of gRNA scaffold. Multiple restriction enzyme target

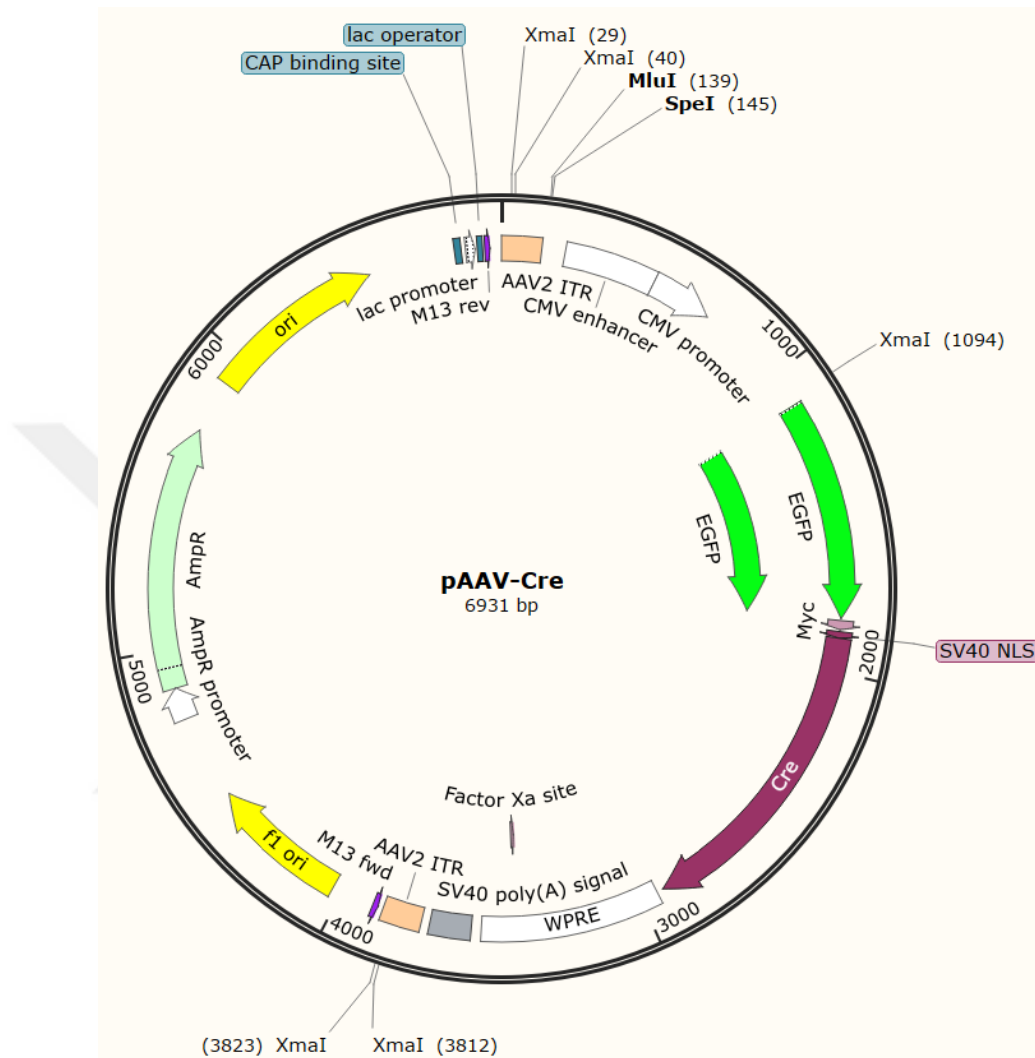


Figure 20. A map of pAAV-Cre plasmid. pAAV-Cre plasmid contains AAV2 ITRs at 5' and 3' ends. Plasmid expression is controlled by CMV promoter and enhancer. It also contains WPRE upstream of 5' ITR.

site insertions were made to the 5' end of both forward and reverse primers to enable further modifications of produced vectors. Vectors carrying single or multiple gRNAs against *Cntf*, *Manf*, *Lif*, and *Fgf* were produced using this strategy. The initial sgRNA to be cloned was amplified from its original vector using a forward primer with MluI, XhoI and EcoRI digestion sites inserted to the 5' and a reverse primer with SpeI and BglII digestion sites inserted to the 5' end. The resulting PCR products were cloned into the pAAV-Cre vector. Single cutter XhoI, EcoRI, SpeI digestion sites are introduced into the resulting AAV-sgRNA vector. It is possible to add 3 more sgRNAs into this vector

through MluI-XhoI, XhoI-EcoRI, and BglII-SpeI double digestions. It is also possible to remove the initial sgRNA by EcoRI-BglII double digestion. However, it is not ideal to clone more than 3 sgRNAs, together with their respective U6 promoter and gRNA scaffold regions, into the pAAV-Cre vector due to size limitations of AAVs. Although it is possible to package vectors up to 5,2 kb, the expression levels are reduced inversely proportional to the vector size. Moreover, vectors exceeding the 5,2 kb limit are truncated on the 5' end [56, 125]. Thus, it is ideal to use an AAV vector that is at most 5 kb in size. We cloned 2 sgRNAs into the pAAV-Cre vector and produced an AAV2/ShH10 with a high titer ($8,3 \times 10^{11}$ vg/ml), showing the effectiveness of the multiple sgRNA cloning strategy.

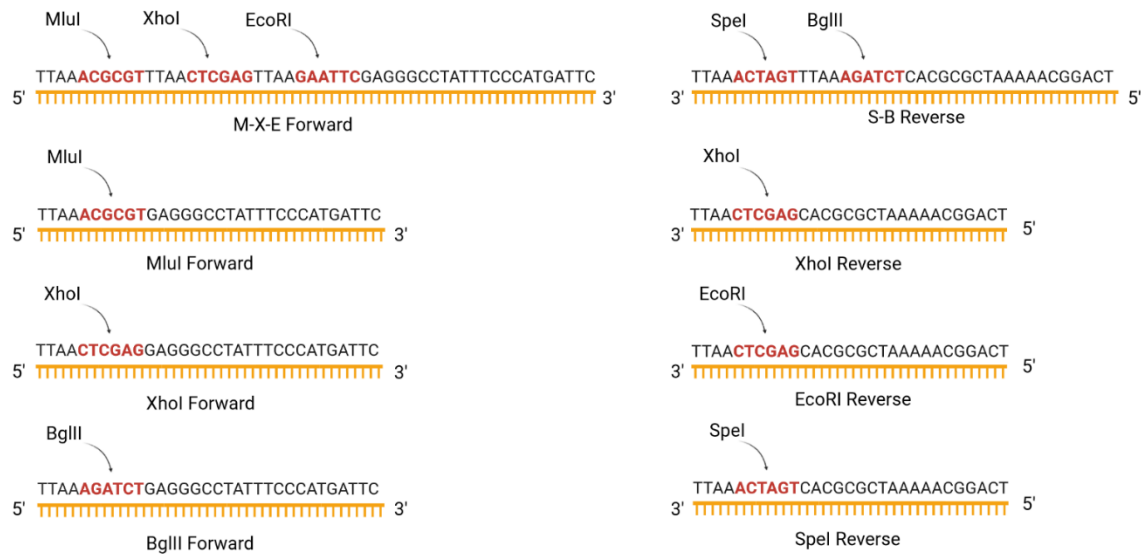


Figure 21. Cloning strategy for pAAV-sgRNA. Forward primers targeting the 5' of U6 promoter and reverse primers targeting the 3' of gRNA scaffold were designed. 5' of the forward primers were modified to contain either MluI, XhoI, and EcoRI together (M-X-E forward) or one of MluI (MluI forward), XhoI (XhoI forward), BglII (BglII forward). 3' of the reverse primers were modified to contain either SpeI and BglII together (S-B reverse) or one of XhoI (XhoI reverse), EcoRI (EcoRI reverse), SpeI (SpeI reverse). The first sgRNA insert was amplified using MXE forward and SB reverse.

3.2.2. Confirmation of dCas9-SPH-Mediated Overexpression *in vitro*

A dCas9-SPH Müller glia cell line was produced in our lab by dissociating retinas from dCas9-SPH animals and incubating them in cell culture dishes. We switched from our previous dCas9-VP64 cell culture system to the dCas9-SPH cells to precisely test the

pAAV-sgRNA system *in vitro*. dCas-SPH cells were transfected with pAAV-sgRNA vectors containing the highest performing sgRNAs from our previous screens. These cells were sorted according to their GFP expression. cDNA libraries were produced and ddPCR was performed. ddPCR results indicated a significant overexpression of *Lif* (16-fold), *Cntf* (12-fold), *Manf* (2-fold), and *Fgf2* (10-fold) genes (unpublished data). Such a significant overexpression shows the effectiveness of the CRISPR-SpdCas9-SPH system *in vitro*. The overexpression levels will be inevitably reduced *in vivo*, and lower titers of AAV can be administered to control the dosage. *Manf* overexpression was limited to 2-fold due to inherently high expression levels of *Manf*.

3.3. AAV-sgRNA Titration

As the efficiency and efficacy of the designed sgRNAs were confirmed *in vitro*, *in vivo* delivery of this system was done with the best performing sgRNAs. AAVs were produced using the AAV2/ShH10 capsid. AAV2/ShH10 capsid was produced through directed evolution to specifically target Müller glia cells in the mouse retina when injected intravitreally [66, 126, 127]. AAVs were produced using the large-scale production protocol and purified from an iodixanol gradient. An AAV containing the U6 promoter and gRNA scaffold without a sgRNA was produced (AAV2/ShH10-Ctrl) as a negative control. Primers targeting WPRE were employed instead of ITR to obtain the absolute amount of successfully packaged AAV genome. We obtained very high titers in our initial AAVs between the range of 10^{11} - 10^{12} vg/ml.

Table 18. Titers of AAV-sgRNA. AAV-sgRNA titration results.

AAV	Capsid	Titer (vg/ml)
AAV2/ShH10-Ctrl	ShH10	$1,88 \times 10^{12}$
AAV2/ShH10-LIF7	ShH10	$1,71 \times 10^{12}$
AAV2/ShH10-MANF10	ShH10	$6,68 \times 10^{11}$
AAV2/ShH10-FGF2	ShH10	$2,82 \times 10^{11}$
AAV2/ShH10-CNTF7	ShH10	$3,08 \times 10^{11}$

3.4. AAV-sgRNA Cre Recombinase Activity

Initially, we tested the transduction rate and expression profile of the AAV-sgRNA vector by observing the fluorescent signal due to Cre recombinase and GFP expression. An Ai14 mutant animal was injected with 2 μ l of AAV2/ShH10-LIF7 ($1,71 \times 10^{12}$ vg/ml) on the left eye and AAV2/ShH10-Ctrl ($1,88 \times 10^{12}$ vg/ml) on the right eye at postnatal week 7 (**Fig.22**). It was bright-field and fluorescent fundus imaged before injection and 10 days post-injection. GFP signal was not detected and some tdTomato leakage was present due to Ai14 before injection. At day 10 post-injection, there was an intense amount of GFP and tdTomato signal in the retina. We confirmed with this study that the AAV-sgRNA successfully transduces retinal cells, and expresses Cre recombinase and produces GFP signal.

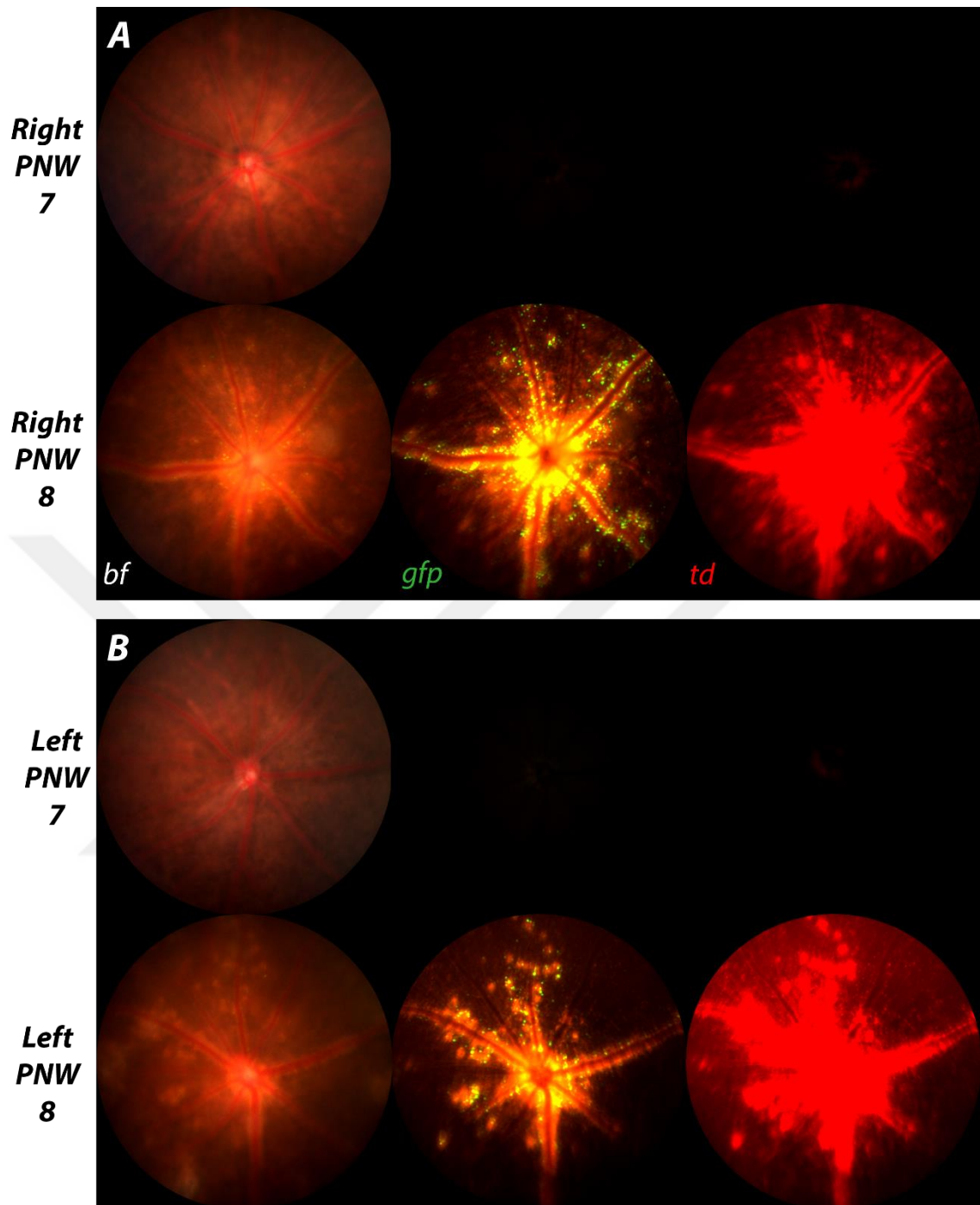


Figure 22. Testing the *in vivo* Cre recombinase activity of AAV-sgRNA. An Ai14 transgenic animal at PNW 7 was injected with 2 μ l of **A)** AAV2/ShH10-Ctrl (1.71×10^{12} vg/ml) on the right eye and **B)** AAV2/ShH10-LIF7 (1.88×10^{12} vg/ml) on the left eye. GFP and tdTomato fluorescence activities, indicating Cre recombinase-mediated recombination of stop codon, were observed at PNW 8 in both eyes. Bright-field images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP and tdTomato separately.

3.5. dCas-SPH Mice Characterization

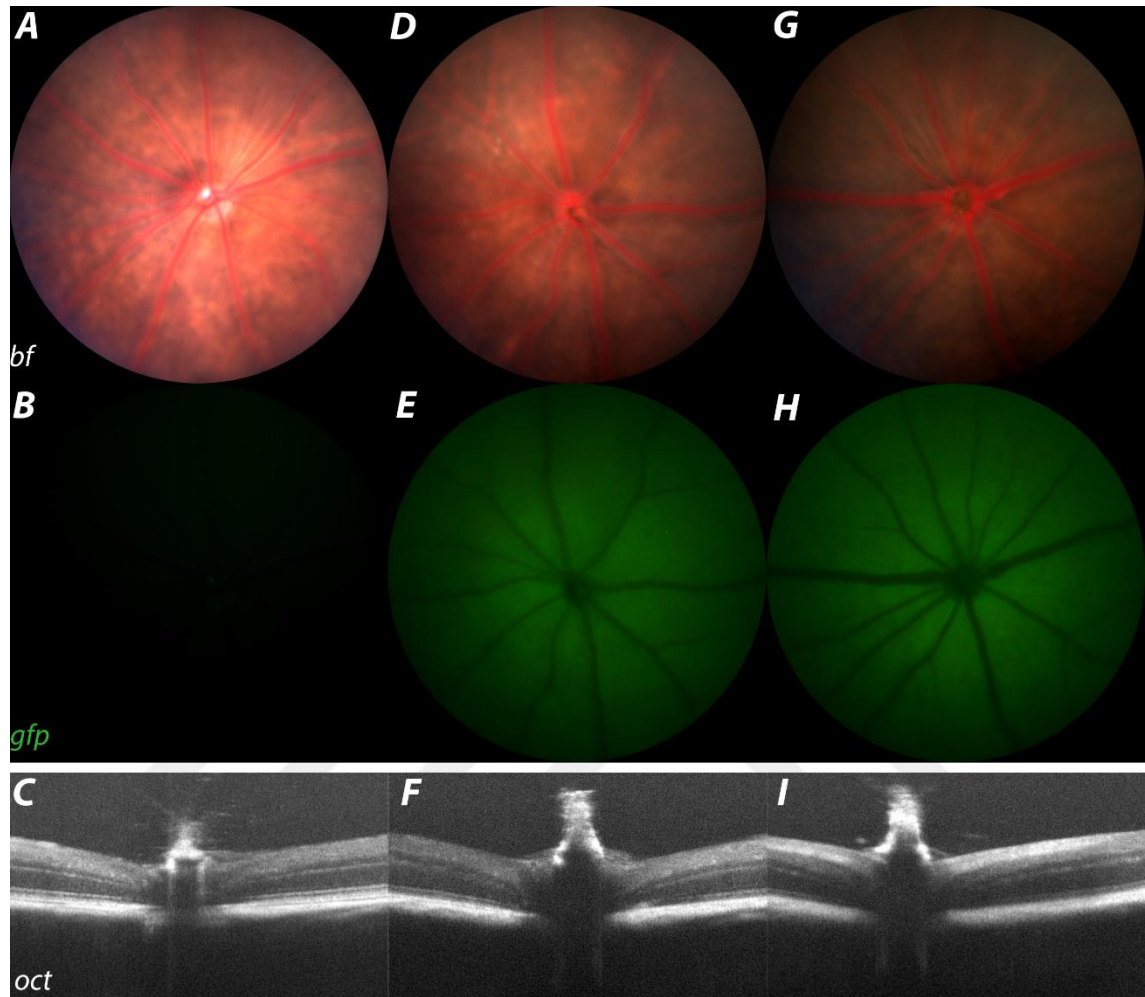


Figure 23. Fundus features of dCas-SPH mice. *D, G) Bright-field fundus and F, I) OCT images of dCas-SPH animals are highly similar to A, C) C57BL/6 animals. E, H) A leaky GFP expression can be observed in high exposure settings in fluorescent fundus images in comparison to B) C57BL/6. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP.*

We performed intravitreal AAV-sgRNA injections to dCas-SPH animals to test the *in vivo* overexpression capabilities of this system. Prior to intravitreal injections, we performed bright-field and fluorescent fundus, and OCT imaging to assess the retinal morphology of dCas-SPH animals. dCas-SPH animals express Cre recombinase-dependent Spd-Cas9-SPH as well as GFP. However, we have shown that dCas-SPH animals have leaky GFP expression even in the absence of Cre recombinase (**Fig.24**). Although the pAAV-sgRNA vector contains GFP as well, this was not a serious problem as pAAV-sgRNA exhibits stronger GFP expression levels compared to the leaky GFP expression (**Fig.25**).

3.6. *In vivo* Activity of dCas-SPH

Viral dose assessment is critical for viral vector-mediated gene therapy products. Thus, we performed intravitreal injections to dCas-SPH animals that do not show retinal degeneration phenotype to find the most ideal AAV-sgRNA dose range. Moreover, these injections will serve as proof-of-concept to confirm the *in vivo* overexpression capabilities of the CRISPR-dCas9-SPH system. Initially, we tested this system on dCas-SPH (PNW 20) and SPH rod reporter (Ai14; Tg(Rho-Cre); dCas-SPH) (PNW 29) animals. Retinal detachment can be seen in the d0 fundus images of both animals (Figure.25 A, B, C, D). Animals were bilaterally injected with 2 μ l each of AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) on the right eye and AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) on the left eye. At PNW 30, there was excessive GFP and tdTomato expression in both eyes of the SPH rod reporter mouse (**Fig.25 A, B**). SPH rod reporter mouse had retinal blur, especially in the left eye, in PNW 30 that increased significantly in PNW 33. This retinal blur seems to affect the fluorescence intensity in the retina (**Fig.25 A**). dCas-SPH animal had retinal detachment or oedema in both eyes after intravitreal injection. The area of retinal detachment was reduced by PNW 24 in both eyes (**Fig.25 C, D**). The excessive fluorescent signals were visible in the bright-field fundus images as well.

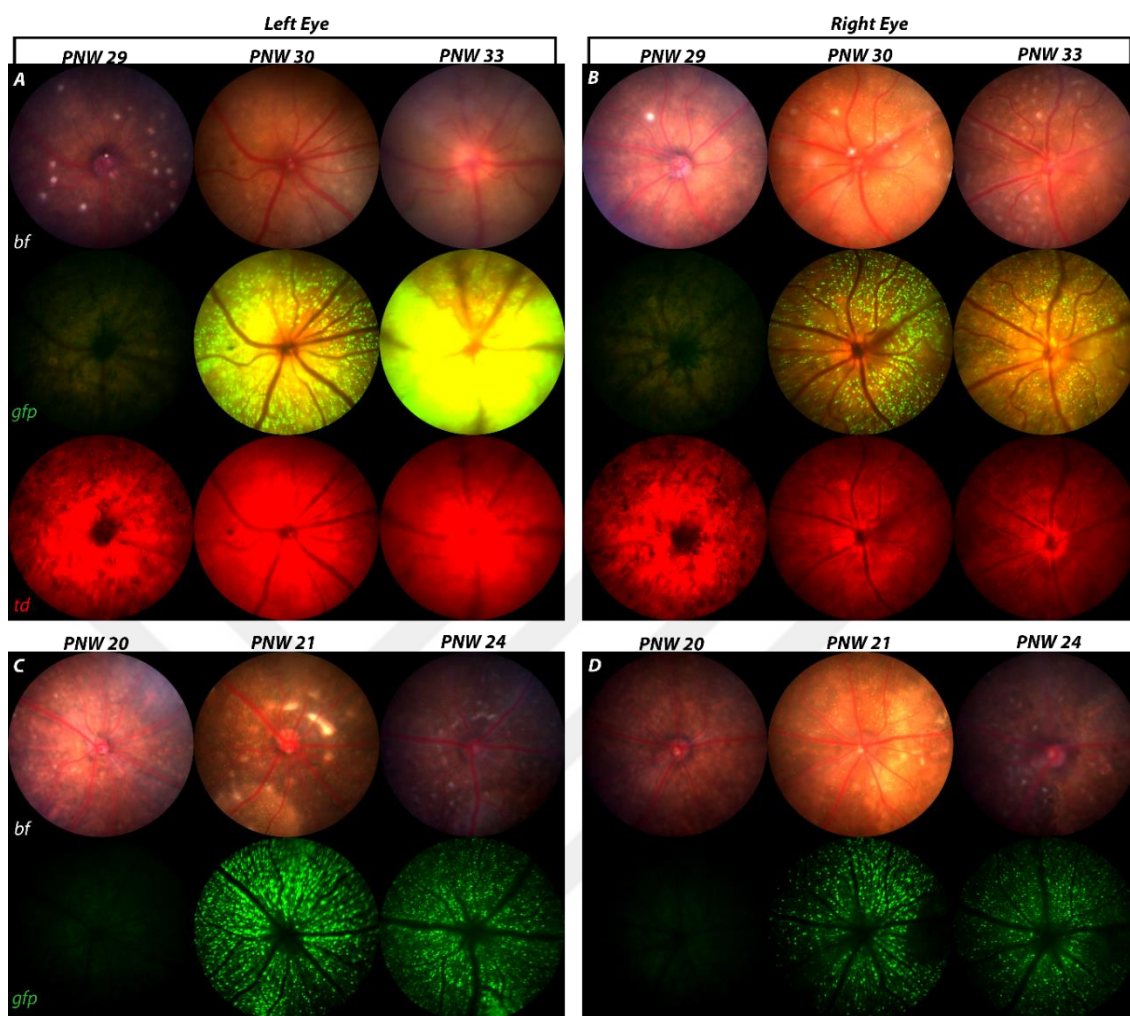


Figure 24. AAV-sgRNA-mediated in vivo dCas-SPH activation. An SPH rod reporter mouse was injected with 2 μ l of AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) on the **A)** left eye and AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) on the **B)** right eye. **A)** A GFP leak can be observed due to leaky dCas-SPH expression before injection. tdTomato fluorescent signal also leaks into the GFP channel, producing a yellow color. An intense tdTomato signal indicates the millions of rod photoreceptors in the healthy retina. A slight blur is visible in all channels at post-injection week 1. The blur increases at post-injection week 4. GFP signals are concentrated in Müller cells due to AAV2/ShH10 capsid tropism. tdTomato signal is dissipated throughout the retina due to secretion of Cre recombinase to nearby cells. **B)** A small amount of blur, although less than left eye, is also visible on the right eye at post-injection weeks 1 and 4. **C)** A dCas-SPH mouse was injected with 2 μ l of AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) on the **C)** left eye and AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) on the **D)** right eye. **C, D)** Transduced cells are clearly visible compared to SPH rod receptor eyes. Retinal blur is absent from both **C** and **D**. A region with retinal damage can be observed in the temporal side of the right eye. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP and tdTomato separately.

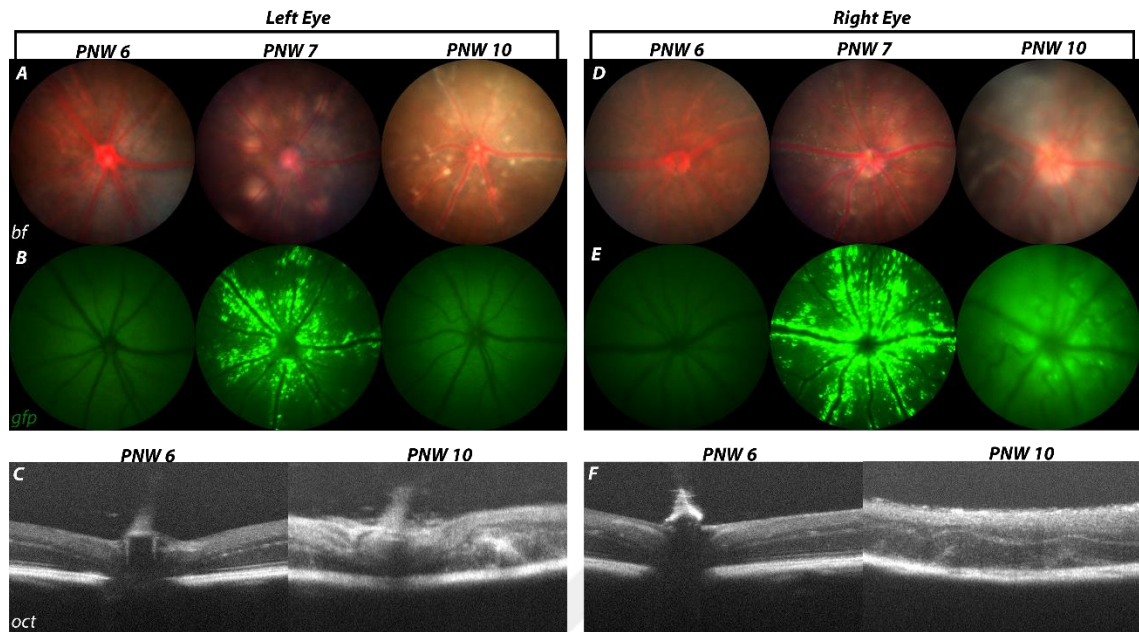


Figure 25. AAV-sgRNA in vivo safety test. dCas-SPH animals ($n=4$) were intravitreal injected with $2 \mu\text{l}$ AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) to their **A, B, C** left eyes and AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) to their **D, E, F** right eyes. This representative figure shows the initial retina at PNW 6, and one week after injection at PNW 7 and 4 weeks after injection at PNW 10. **A, B, D, E**) Retinal detachment and blur are present in both left and right eyes. **B, E**) GFP expression reached its peak at PNW 7-8. GFP signal was almost completely lost in all eyes at PNW 10. **C, F**) Retinal detachment sites are also visible in OCT images at PNW 10. Retinal layer disorganization was observed in OCTs of both eyes. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP.

We wanted to confirm our initial findings and further assess the effects of intravitreal AAV-sgRNA. We established our OCT imaging system, that would help us understand the effects of intravitreal AAV-sgRNA injection on retinal layers, in the meantime as well. Thus, a set of dCas-SPH animals were injected with AAV-sgRNA at post-natal week 6 (PNW 6). Animals were injected bilaterally into the vitreous with $2 \mu\text{l}$ each of AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) to their left eyes and AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) to their right eyes. Animals were imaged biweekly after the injections until 6 weeks after injection. Successful GFP expression was observed in all retinas, however, the GFP signal was lost in all retinas at PNW 10 (**Fig.26 B, E**). GFP signal was lost after PNW 7 in one animal, PNW 8 in one animal, and PNW 9 in two animals post-injection. Retinal detachment and blur was observed in fundus images after PNW 8 (**Fig.26 A, D**). OCT images indicated retinal detachment, and disorganization of inner retinal layers due to inflammation and immune reaction (**Fig.26 C, F**). Retinal layer disorganization was observed in 3 right retinas while in 2 left retinas. The presence of

complications in control retinas indicates that they might not be caused by gene overexpression-mediated toxicity. We hypothesized that this was due to the AAV titer, and we performed further injections with varying AAV-sgRNA doses to find the safest dose range.

3.7. Rd10-SPH Follow-up

We performed a followup study to characterize the retinas of dCas-SPH expressing rd10 animals. rd10 homozygous x dCas-SPH animals were bred to obtain rd10 heterozygote and dCas-SPH expressing offspring. This first generation offspring were inbred to obtain rd10 homozygote and dCas-SPH expressing (rd10-SPH) animals. The GFP leakage found in dCas-SPH retinas was a concern and we wanted to compare the retinas of rd10 and rd10-SPH animals through this study. We imaged rd10-SPH animals (n=2) biweekly from postnatal week 3 to week 7. Background GFP levels were steady throughout the follow-up period. These results indicate that degeneration profile and retinal morphology of rd10-SPH mice are almost identical to rd10 mice (**Fig.27**).

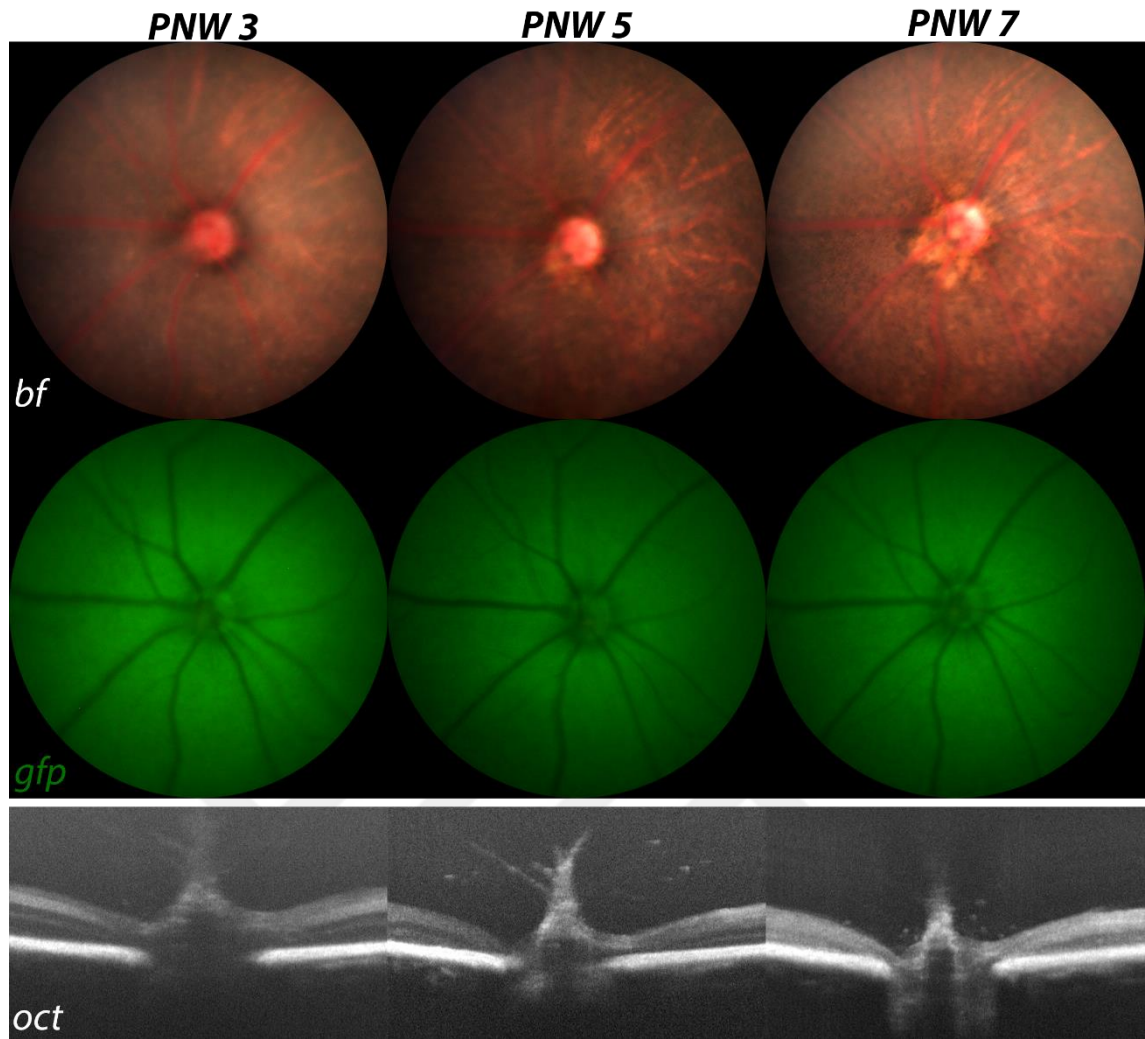


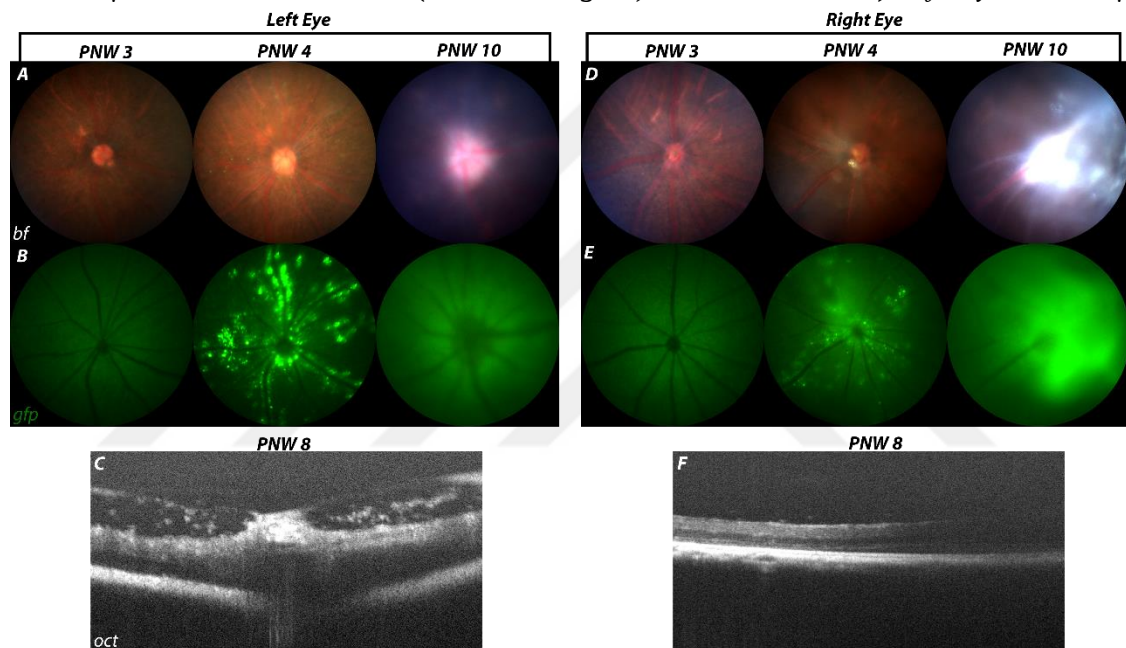
Figure 26. Characterization of Rd10-SPH mice retina. Rd10-SPH mice were imaged biweekly from PNW 3 to PNW7 with fluorescent and bright-field fundus and OCT imaging. Retinal degeneration around optic nerve and feature of atrophic retina such as vessel attenuation was observed in fundus images. Leaky GFP expression due to dCas-SPH was observed in fluorescent fundus images. ONL thickness changes were in line with the rapid atrophy of rod cells reported in the literature [50]. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP.

3.8. dCas-SPH Activity in a Fast Degeneration Mouse Model

Rd10-SPH animals were injected bilaterally with 2 μ l AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) on the left eye and 2 μ l AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) on the right eye. PNW 3 rd10-SPH animals were chosen for injection as the retinal responses are at peak during this period. Injected animals were imaged until PNW 10. 1 mouse was excluded

from the study at PNW 4 as it showed symptoms of developmental delay. 2 left retinas had almost no GFP expression throughout the study while only 1 retina successfully showed GFP expression (**Fig.28**). 3 right eyes developed cataract by PNW 5 due to inflammation. 2 left retinas with no GFP expression remained normal throughout the study, while the third left retina with GFP expression showed signs of cataract and inflammation by PNW 5. We found this dose level to be toxic in the fast degeneration mouse model as well. Thus, we performed another set of injections with half the volume of AAV to reduce complications.

Figure 27. Viral load testing in Rd10-SPH retinas. Rd10-SPH mice (n=4) were injected with 2 μ l AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) on the **A, B, C** left eye and 2 μ l



AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) on the **D, E, F** right eye. One animal was excluded from the study PNW 4 due to severe retinal damage. **A, D**) Three right eyes and one left eye experienced severe inflammation by PNW 8 following AAV-sgRNA injections. **C, F**) OCT images at PNW 8 reflect inflammation as well. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP.

Another rd10-SPH cohort was injected with 1 μ l each of either AAV2/ShH10-LIF7 (1.71×10^{12} vg/ml) or AAV2/ShH10-LIF7 and AAV2/ShH10-CNTF7 (1.01×10^{12} vg/ml) on the left eye, and AAV2/ShH10-Ctrl (1.88×10^{12} vg/ml) on the right eye at PNW 3. AAV2/ShH10-LIF7 and AAV2/ShH10-CNTF7 was coinjected to test the effects of simultaneous activation of multiple neurotrophic factors. A significant improvement in outer nuclear layer thickness was not observed. However, a slight increase was visible in AAV2/ShH10-LIF7 injected retinas, close to significance (**Fig.29 G**). Retinal cells of injected animals were sorted at PNW 7. We wanted to assess if the CRISPR-SpdCas9-

SPH expression affects the retina. We selected a group of rd10 homozygous and rd10-SPH animals, and isolated their retinas at PNW 7. Retinas of C57BL/6 were isolated as well. ddPCR analysis of *Rho* expression levels normalized to *Gapdh* showed no significant increase in *Rho* expression in treated retinas (**Fig.29 H**). All rd10 retinas expressed significantly reduced levels of *Rho*, as most of the ONL is degenerated by PNW 7.

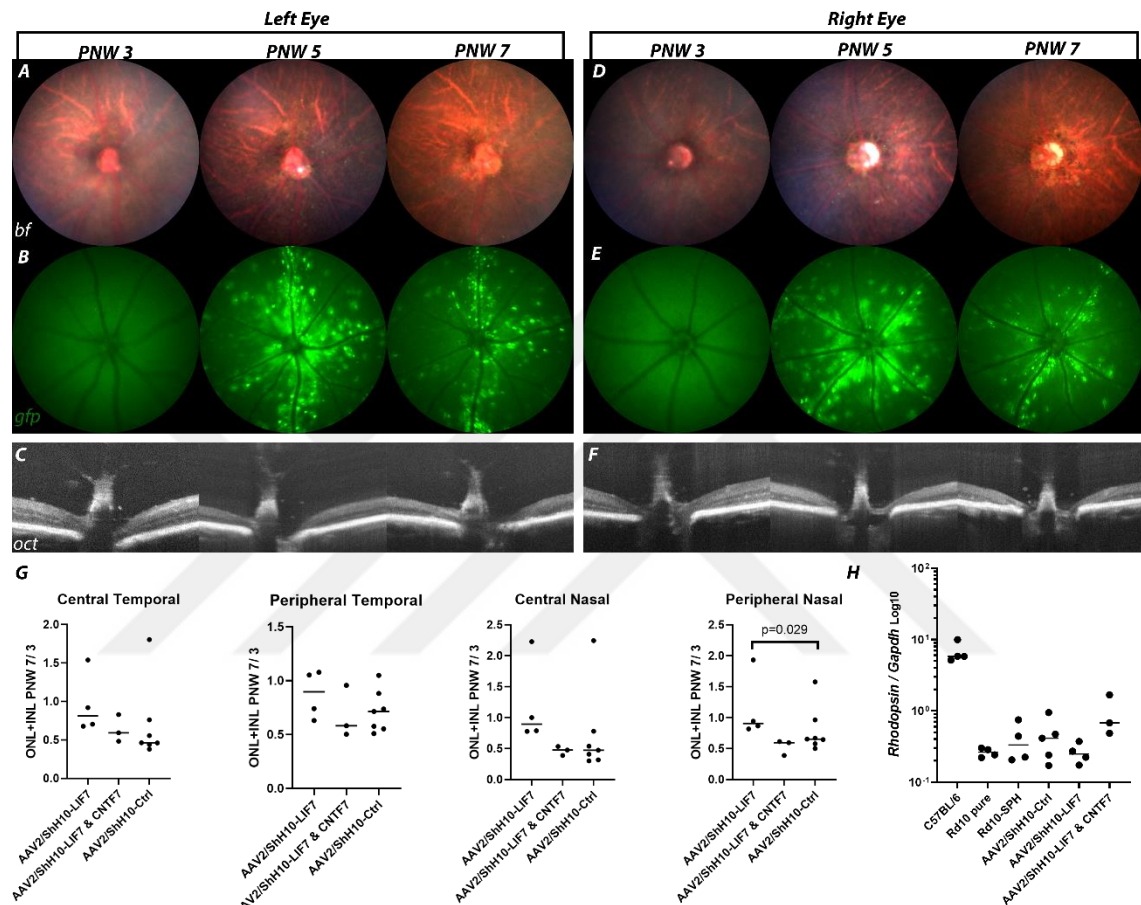


Figure 28. Single and combined AAV-sgRNA in vivo trial. A representative figure showing rd10-SPH animals that were injected 1 μ l of either AAV2/ShH10-LIF7 (n=4, 1,71x10¹² vg/ml) or AAV2/ShH10-LIF7 and AAV2/ShH10-CNTF7 mixture (n=3, 1,01x10¹² vg/ml) on the **A, B, C**) left eyes or AAV2/ShH10-Ctrl (n=7, 1,88x10¹² vg/ml) on the **D, E, F**) right eyes. **B, E**) GFP expression levels were reduced by PNW 7 in both eyes for all animals. **C, F**) OCT images indicate significant atrophy of both left and right eyes in all animals. **G**) OCT images of injected retinas were measured at PNW 3 and PNW 7 to compare the thickness of ONL and INL layers together. A significant increase was not present in injected retinas, although some increase was visible. **H**) Rhodopsin expression levels of C57BL/6, rd10 homozygous, rd10-SPH, and AAV-sgRNA injected rd10-SPH animals were normalized to *Gapdh* expression. A significant increase of *Rho* expression was not found in AAV-sgRNA treated retinas. Multiple ratio-paired t-tests were performed for statistical analysis of **G**. Multiple unpaired two-tailed Mann-Whitney tests were performed for statistical analysis in **H**. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP.

3.9. dCas-SPH Activity in a Slow Degeneration Mouse Model

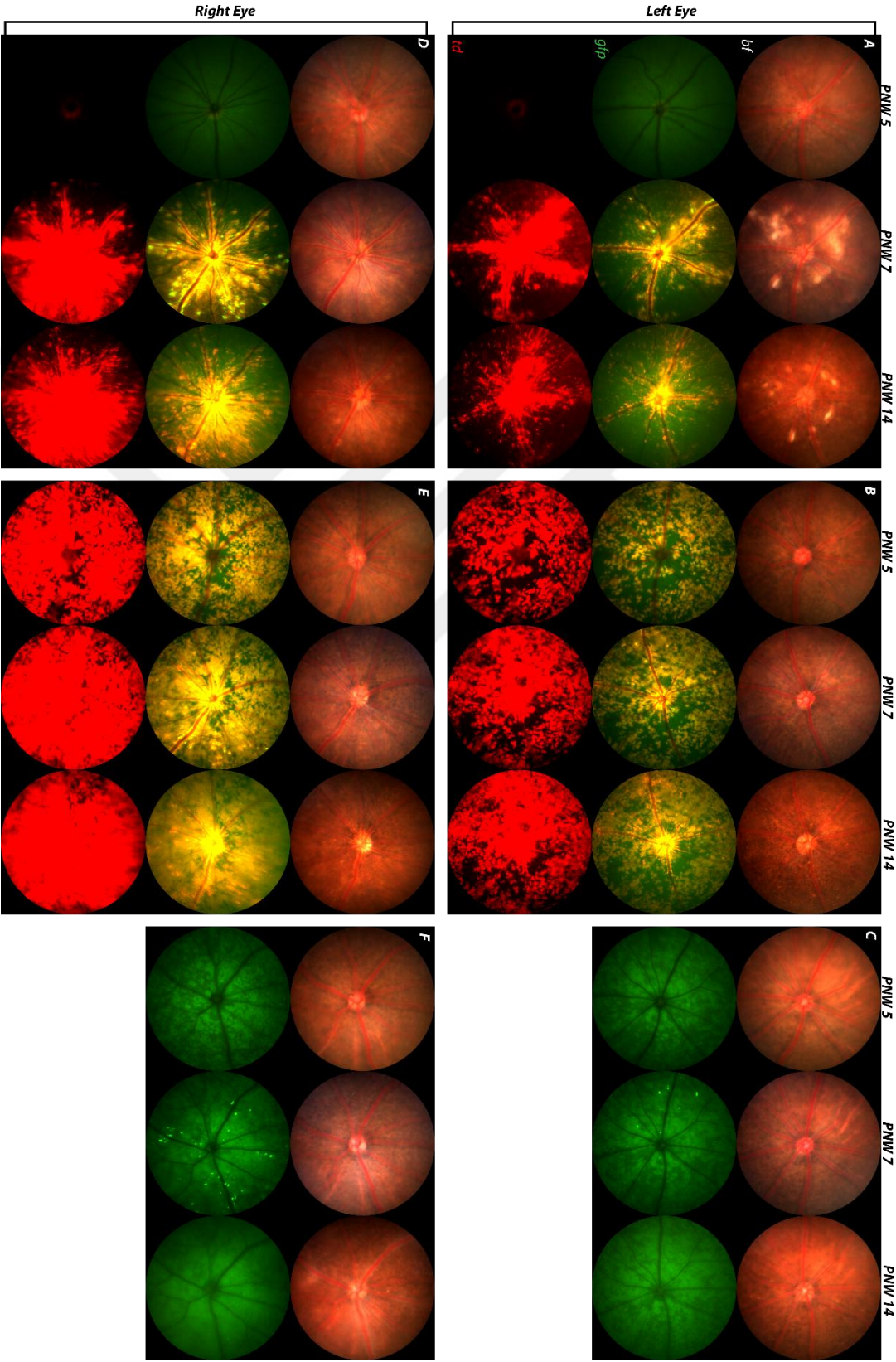


Figure 29. Fluorescent fundus imaging of *Fgf2*, *Manf*, *Lif*, *Cntf* overexpression in a slow degeneration mouse model. P23H-SPH animals (n=5) were injected with an equal titer mixture of 0,5 μ l AAV2/ShH10-LIF7, AAV2/ShH10-MANF10, AAV2/ShH10-CNTF7, AAV2/ShH10-FGF2-7 (AAV2/ShH10-MIX, $7,43 \times 10^{11}$ vg/ml) into the **A**) left eyes and 0,5 μ l AAV2/ShH10-Ctrl ($1,88 \times 10^{12}$ vg/ml) into the **D**) right eyes. Some retinal detachment and inflammation were present in both eyes after injections. Fluorescent intensity for both GFP and tdTomato were slightly reduced by PNW 14. Tg(Rho-Cre) transgenic P23H-SPH animals (n=3) were injected with 0,5 μ l AAV2/ShH10-MANF10 ($6,68 \times 10^{11}$ vg/ml) into the **B**) left eyes and 0,5 μ l AAV2/ShH10-Ctrl ($1,88 \times 10^{12}$ vg/ml) into the **E**) right eyes. tdTomato fluorescence intensity following injections increased compared to the existing tdTomato expression levels due to Tg(Rho-Cre) transgene. GFP fluorescence intensity levels were steady at PNW 14 as well. P23H-SPH animals without the Ai14 transgene (n=2) were injected with 0,5 μ l AAV2/ShH10-FGF2 ($2,82 \times 10^{11}$ vg/ml) into the **C**) left eyes and 0,5 μ l AAV2/ShH10-Ctrl ($1,88 \times 10^{12}$ vg/ml) into the **F**) right eyes. GFP fluorescence levels were lower in left eyes due to reduced titration compared to the right eyes. Inflammation was absent from all retinas at PNW 14. Bright-field fundus images are taken with equal exposure settings. Fluorescent fundus images are taken with equal exposure settings for GFP and tdTomato separately.

Three cohorts of PNW 5 P23H-SPH animals were chosen for AAV-sgRNA treatment. Cohort 1 included P23H-SPH animals that are ai14 transgenic. Cohort 1 was injected with 0,5 μ l of a mix of AAV2/ShH10-LIF7, AAV2/ShH10-MANF10, AAV2/ShH10-CNTF7, and AAV2/ShH10-FGF2 (AAV2/ShH10-MIX, $7,43 \times 10^{11}$ vg/ml) in the left eyes. Cohort 2 included P23H-SPH animals that were ai14 and Tg(Rho-Cre) transgenic. Cohort 2 was injected with 0,5 μ l of AAV2/ShH10-MANF10 ($6,68 \times 10^{11}$ vg/ml) into the left eyes. Cohort 3 included P23H-SPH animals that did not have ai14 or Tg(Rho-Cre) transgenes. Cohort 3 was injected with 0,5 μ l of AAV2/ShH10-FGF2 ($2,82 \times 10^{11}$ vg/ml) in the left eyes. All animals were injected with 0,5 μ l AAV2/ShH10-Ctrl ($1,88 \times 10^{12}$ vg/ml) into the contralateral eye. Retinas were bright-field and fluorescent fundus (**Fig.30**) and OCT (**Fig.31**) imaged biweekly from PNW 5 to PNW 14. None of the retinas showed signs of inflammation and only some of them had retinal detachment or oedema (**Fig.30**). Fluorescence intensity of both GFP and tdTomato reduced only slightly by PNW 14. Signs of mild inflammation were present in some of the retinas by PNW 14 (**Fig.30**). 2 right eyes were excluded from analysis due to retinal complications caused by the injection procedure. One right eye developed atrophy in the central retina unrelated to the injection procedure. ONL and INL combined retinal layer thickness was similar for both eyes in all cohorts (**Fig.30 A-D**). Central left retinas from all cohorts showed a higher trend in ONL, INL thickness without significance (**Fig.32 C, D**). Retinas from all cohorts were isolated and gene expression analysis was performed by ddPCR. Rhodopsin expression was normalized to *Gapdh* for all retinas. A significant increase in rhodopsin

expression in comparison to control retinas was not detected with an unpaired analysis (Fig.32 F). However, comparing rhodopsin expression of left and right eyes in pairs shows a 7% increase of rhodopsin expression in the AAV2/ShH10-MIX injected cohort 1, and a 36% increase of rhodopsin expression in the AAV2/ShH10-FGF2 injected cohort 3 (Fig.32 E).

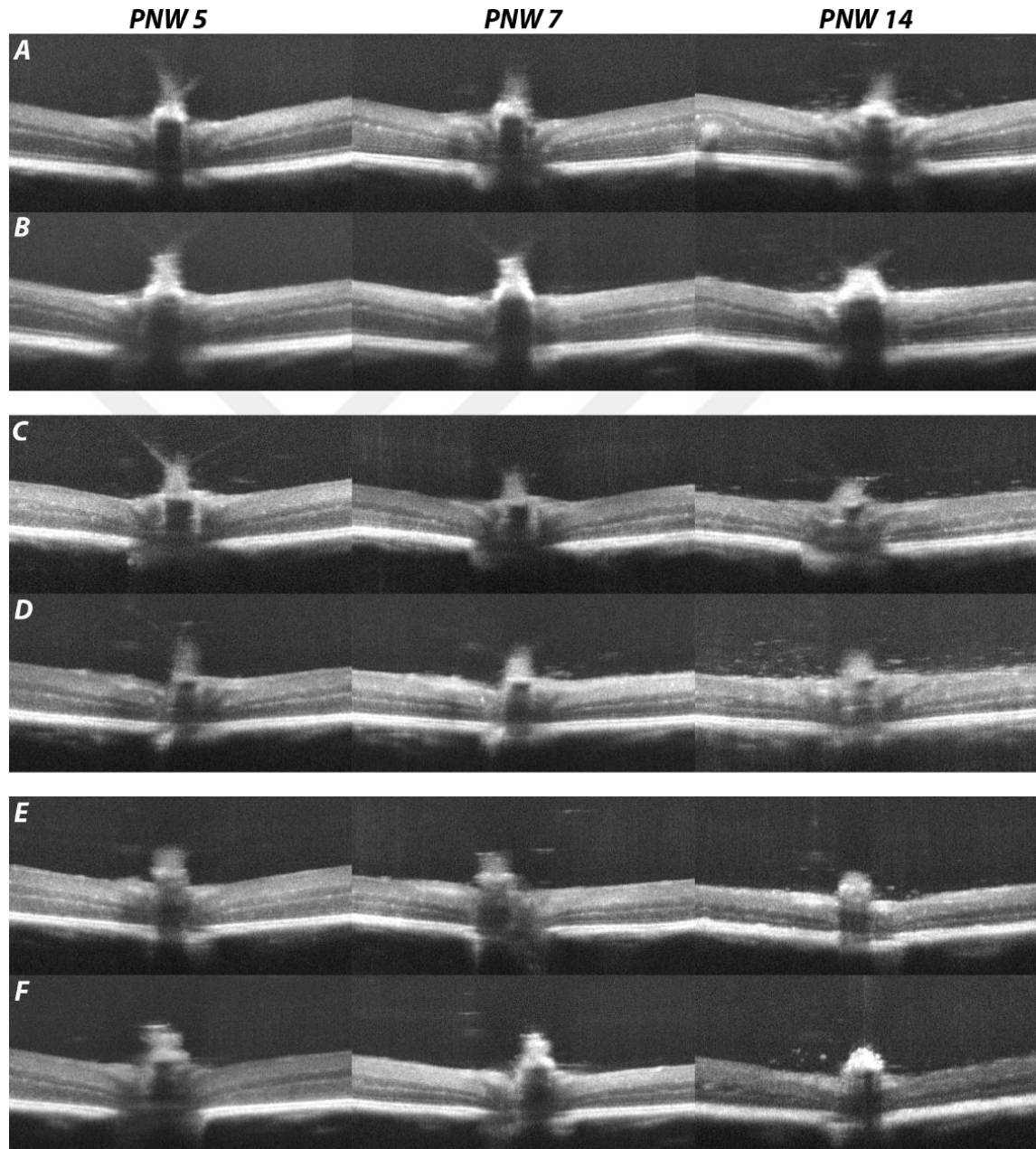
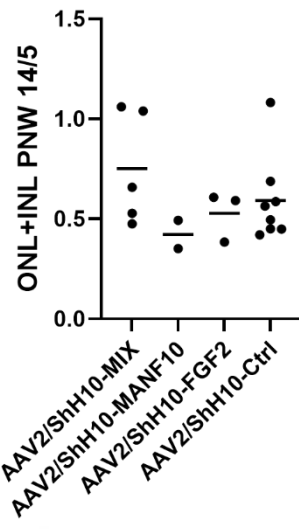
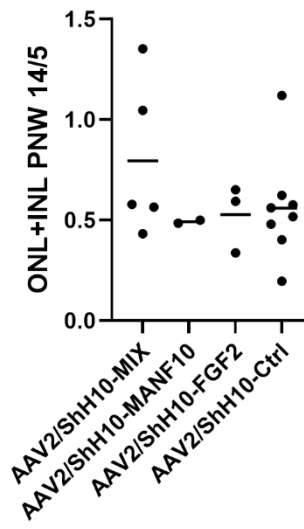


Figure 30. OCT imaging of *Fgf2*, *Manf*, *Lif*, *Cntf* overexpression in a slow degeneration mouse model. OCT images of AAV2/ShH10-MIX injected **A)** P23H-SPH, AAV2/ShH10-MANF10 injected **B)** Tg(Rho-Cre) transgenic P23H-SPH, and AAV2/ShH10-FGF2-7 injected **C)** P23H-SPH without Ai14 animals. **B, D, F)** All right eyes were injected with AAV2/ShH10-Ctrl. Gradual loss of ONL is present in all retinas.

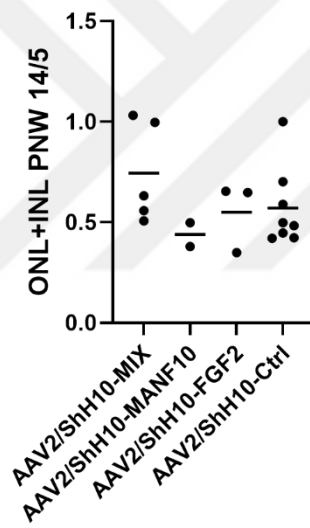
A Peripheral Temporal



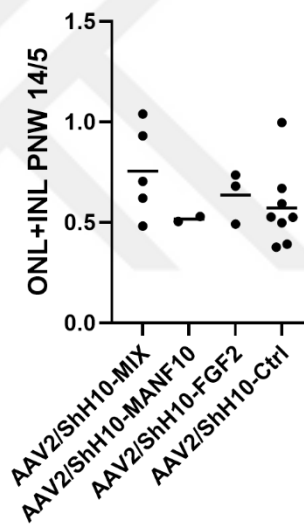
B Peripheral Nasal



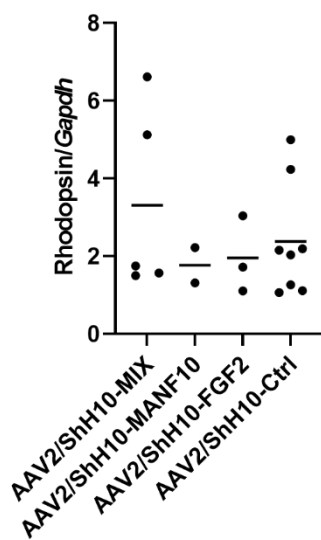
C Central Temporal



D Central Nasal



E



F

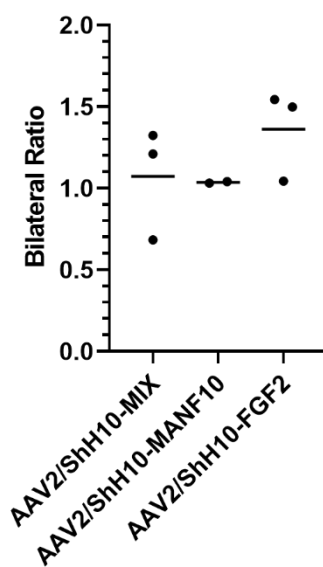


Figure 31. Retinal thickness and rhodopsin expression levels of AAV-sgRNA treated retinas. **A, B, C, D)** A significant difference in the retinal layer thickness of left and right eyes was not found for all treatment groups. **C, D)** Central retina was thicker in all groups, although insignificant. **E)** AAV2/ShH10-MIX and AAV2/ShH10-FGF2 injected left eyes showed 7% and 36% increase in rhodopsin expression, normalized to Gapdh, compared to right eyes. **F)** Rhodopsin expression normalized to Gapdh expression shows that AAV2/ShH10-MIX increases rhodopsin positive cells without significance. Multiple unpaired Welch t-tests were performed for statistical analysis.



4. DISCUSSION

We successfully produced a rod cell reporter retinal degeneration (P23H rod reporter) mouse model in this thesis. P23H rod reporter triple transgenic animals carry the P23H mutation and express transgenes ai14 and Tg(Rho-Cre). The Ai14 transgene carries a tdTomato gene with a floxed stop codon in the *Rosa26* locus. Tg(Rho-Cre) transgene encodes for a Cre recombinase protein under the rhodopsin promoter. Thus, these triple transgenic P23H mutant animals can report the rod cell health through tdTomato fluorescence. We found that the rod photoreceptor population indicated by the fluorescence intensity is at its peak at PNW 6 in P23H rod reporter animals. tdTomato fluorescence reaches its highest point at this time point, indicating the completion of retinal development. A steep decline in rod cell population occurs after PNW 12 where a significant reduction of tdTomato fluorescence is indicated. The P23H rod reporter mouse line became the basis of our future plans for a neuroprotective transcriptional regulation gene therapy. The mild degeneration of P23H heterozygous retinas better reflects the RP progression in humans than homozygous retinas, which show a faster progression of retinal degeneration [48]. This also gives us a window of opportunity for a neuroprotective attenuation of retinal atrophy. A potential neuroprotective therapy needs to be delivered at least 4 weeks before the retinal degeneration reaches its peak. This time is needed for the episomal vector delivered by the AAV to be expressed in cells and for the expressed transcriptional regulatory system to activate the expression of neurotrophic factors [128]. Studies show that vector expression following AAV transduction *in vivo* can take several weeks to reach its maximum state. The duration of this delay changes according to the composition of the AAV vector, the serotype of the AAV and the target organism [129, 130]. Thus, we determined the ideal injection time for each mouse model considering these factors.

We originally sought to use a dCas9-VP64 transgenic mouse line for the *in vivo* transcriptional regulation study. However, we encountered problems due to the pandemic and could not produce this transgenic mouse line. A dCas9-VP64 stable cell line was produced previously and we found efficient overexpression of neurotrophic factors *in vitro*. We decided to move on to another transgenic line, called dCas-SPH. Studies show a higher efficiency of transcriptional regulation with dCas-SPH compared to dCas9-VP64 system [105]. Although it was a drastic change in our experimental plans, we managed to get results with this new system. A dCas-SPH stable cell line was produced in our lab and

we found an immense overexpression of neuroprotective genes *Lif*, *Cntf*, *Manf*, *Fgf2* (unpublished data). We received results that were supporting this as dCas9-SPH achieved up to 6-fold higher overexpression capabilities *in vitro* compared to the dCas9-VP64 system (unpublished data). *Manf* overexpression was comparably lower than other neurotrophic genes and we are assuming that it is caused by the high basal expression levels of this gene (unpublished data).

After the characterization of the rod cell reporter retinal degeneration mouse model, we designed a cloning strategy for sgRNAs to be used with CRISPR-dCas9-SPH system. We produced a cloning system that would enable us to insert up to 3 sgRNAs into a single AAV. This highly modular vector could be modified to remove or insert different combinations of sgRNAs with ease. The only limiting factor with this cloning strategy is the size of the AAV. It is only possible to package 3 sgRNAs into an AAV due to size constraints. Additionally, we confirmed the Cre recombinase and GFP expression activities of pAAV-sgRNA *in vitro* using a transgenic cell line expressing a tdTomato gene with a floxed stop codon (unpublished data).

We experienced leaky fluorescent protein expression in animals that were either ai14 or dCas-SPH transgenic. tdTomato fluorescent signal was observed in ai14 animals at very high exposure levels. GFP signal was observed at very high exposure levels in dCas-SPH animals as well. Interestingly, the leaky tdTomato expression from the ai14 animals were present throughout the retina, but it was more intense around the optic nerve. Ai14 and dCas-SPH animals contain floxed stop codons that hinder the expression of fluorescent proteins. The leaky expression of tdTomato in ai14 animals is documented by The Jackson Laboratory. This expression prior to Cre recombinase exposure is very low in comparison to Cre recombinase activated expression [131], however, no documentation on this property of dCas-SPH animals was reported previously. This GFP expression might be the result of the two component design of the dCas-SPH system. We are assuming that the scFv-p56-HSF1-GFP component connected to the dCas9-SPH system by a P2A linker might be expressed in low concentrations and causing a background GFP expression. Furthermore, The Jackson Laboratory recently reported that the transgene insertion site of dCas-SPH animals is at intron 3 of the *Dpysl2* gene on chromosome 14 [132], in contrast to the *Gm33925* on chromosome 3 reported by the originating article [105]. We experienced high mortality rates in the newborns when crossing dCas-SPH animals with the GFAP-Cre mouse line. Moreover, the dCas-SPH mouse line itself

usually showed higher mortality rate of newborns compared to other transgenic lines. Thus, we believe the characterization of ai14 and dCas-SPH fundus were vital in our experimental strategies. We also characterized the fundus of rd10-SPH animals over four weeks and we did not observe dissimilarities to rd10 retinas (**Fig.27**).

We have previously established a large-scale AAV production system in our lab and produced high titer AAVs. AAV-sgRNAs with high titers were produced through the same process. We initially wanted to test the Cre recombinase expression and activity of the AAV-sgRNA *in vivo*. An ai14 transgenic mouse was injected bilaterally with AAV-sgRNA. Activation of tdTomato expression due to the removal of the floxed stop codon by Cre recombinase was observed in fluorescent fundus imaging. Interestingly, an intense GFP signal that was leaking into the bright-field images was found. This can be caused by nuclear expression of GFP due to Cre recombinase and the AAV vector itself. Then, we tested AAV-sgRNA in dCas-SPH and SPH rod reporter animals. We selected a small cohort initially to observe the affects of high dosage AAV-sgRNA in the retina (**Fig.25**). As we did not encounter any severe effects by four weeks post-injection, we went on with a larger cohort of dCas-SPH animals that were injected with AAV2/ShH10-LIF7 (**Fig.26**). Initially, we achieved successful GFP expression in both retinas, indicating the expression of Cre recombinase. However, some animals were showing signs of mild inflammation in both eyes. GFP signal was completely lost at four weeks post-injection, excluding the background dCas-SPH leak, in all retinas by PNW 10 and a severe inflammation was present in both retinas of most animals. Although inflammation was an expected outcome, these results were quite surprising as this amount of inflammation was unexpected for an intravitreal AAV-delivery of a gene editing tool. The presence of these complications in control retinas indicates that they might not be caused by gene overexpression-mediated toxicity. Mild inflammation due to intravitreal AAV injections are reported in preclinical and clinical studies. A mild immune response can accompany the inflammation as well. The AAV serotype, promoter and AAV vector composition can have an impact on the severity and longevity of the inflammatory response. Evaluation of inflammatory responses requires detailed quantitative analysis, which is not a common practice [133, 134].

We did a trial with the same titer of AAV-sgRNA in a cohort of rd10-SPH animals. Rd10-SPH injections were performed at PNW 3 to enable neuroprotection before the whole ONL atrophies. However, such an early injection timepoint proved challenging as retinal

damage occurred in one retina and one animal experienced developmental delay for unknown reasons. In addition, only 1 left retina had the expected GFP signal in the retina. Two right retinas developed severe inflammation by PNW 10. Reducing the viral load in the next rd10-SPH cohort reduced the signs of severe inflammation, however, GFP signal was lost 2-3 weeks after injection. A significant change in rod cell population was not found between treated and untreated eyes (**Fig.29**). We hypothesized that either the cells, mainly Müller cells, expressing the activated dCas9-SPH are dying over time due to cytotoxicity, or the GFP expression is lost due to CMV inactivation. Complete inactivation of CMV promoter after four weeks is reported in the literature [135, 136]. Thus, due to either of these factors, the expression of neuroprotective factors are reduced to basal levels and retinal degeneration occurs. Performing a cell death assay to control and treated retinas would enable the assessment of apoptosis in dCas-SPH retinas.

We then wanted to test AAV-sgRNA in a slow degeneration model based on the P23H rod reporter mouse model. We very recently started the production of P23H-SPH animals, thus, it took a long time to obtain a consistent birth of P23H animals with dCas-SPH and ai14 transgenes. Our initial crossings resulted in very low chances of obtaining P23H-SPH animals with the desired genotype. In spite of this problem, we performed a P23H-SPH experiment with 3 different cohorts. Severe or medium inflammation were not present in all of the cohorts. However, GFP expression reduced or vanished by PNW 14 in all cohorts. A significant overexpression of neuroprotective genes or increase of ONL was not achieved. We already reduced the AAV titers injected into the vitreous to 25%. Reducing the AAV titer furthermore would be problematic due to the dilution of AAV in the vitreous. Furthermore, inflammation was absent from our low dose cohorts. This suggested that CMV inactivation is possibly impairing the overexpression capabilities of the dCas9-SPH system *in vivo*. We can replace the CMV promoter or add another promoter that will support its function into the pAAV-sgRNA vectors in the future as it was reported that CMV promoter function can be sustained by the support of another promoter [136]. Another option would be to change to another CRISPR-dCas9 model such as the dCas9-VPR or dCas9-SAM, which achieve similar overexpression efficiency to dCas9-SPH [104, 105]. Most importantly, we will need to test the AAV-sgRNA system in more cohorts to analyze its effects *in vivo*. Performing a TUNEL assay, measuring ERG responses and checking for the expression of inflammatory pathways could be important in troubleshooting and understanding the effects of AAV-sgRNA. We believe

that the results from this thesis will be contributing to the use of novel artificial transcriptional factors for the transcriptional regulation of neuroprotective factors *in vivo*.



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6. APPENDIX

Table 19. Cloning primers. Primers for pAAV-sgRNA clonings.

Name	Direction	Sequence
U6 Promoter F	Forward	gagggcctatttcccatgattc
gRNA Scaffold R	Reverse	cacgcgctaaaaacggact
MXE F	Forward	ttaaacgcgtttaactcgagttaagaattcgagggcctatttcccatgattc
SB R	Reverse	ttaaactagtttaaagatctcacgcgctaaaaacggact
M F	Forward	ttaaacgcgtgagggcctatttcccatgattc
X R	Reverse	ttaactcgagcacgcgctaaaaacggact
X F	Forward	ttaactcgaggagggcctatttcccatgattc
E R	Reverse	ttaagaattccacgcgctaaaaacggact
B F	Forward	ttaaagatctgagggcctatttcccatgattc
S R	Reverse	ttaaactagtcacgcgctaaaaacggact

Table 20. Genotyping primers. List of primers for genotyping.

Name	Forward Primer	Reverse Primer
Ai14 mutant	ctgttctgtacggcatgg	ggcattaaagcagcgatatcc
Ai14 wild-type	aaggagctgcagtggagta	ccgaaaatctgtgggaagtc
P23H	ggtagcactgttgggcatct	gacccacagagacaagctc
Rd10	ctttctattctctgtcagcaaagc	catgagtagggtaaacatgggtctg
dCas-SPH	caccatctccctgctgaca	ctgaacttgtggccggttac