

GENE EXPRESSION CHANGES IN DYING PHOTORECEPTORS

by

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LIST OF ABBREVIATIONS

7DCH	7-dehydrocholesterol
7KC	7-ketocholesterol
7KChol	7-ketocholesterol
A	Axoneme
ABCA	ATP Binding Cassette Subfamily A Member
ABCG	ATP-binding cassette subfamily G member
Abs	Absorbance
Ac-a-Tub	Acetylated alpha tubulin
ACAT	Acyl-CoA cholesterol acyltransferase
ACHM	Achromatopsia
Ackr3	Atypical Chemokine Receptor 3
ACOT	Acyl-CoA Thioesterase
Acs14	Acyl-CoA Synthetase Long Chain Family Member 4
Acta2	Actin Alpha 2
AFM	Atomic force microscopy
AIF	Apoptosis-inducing factor
AMD	Age-related macular degeneration
Angptl4	Angiopoietin Like 4
APO	Apolipoprotein
Aqp5	Aquaporin 5
ATF	Activating transcription factor
Atf3	Activating Transcription Factor 3
Axin2	Axin 2
BAX	BCL2-associated X protein
BB	Basal body
BBB	Blood-brain barrier
bFGF	Basic fibroblast growth factor
BrdU	Bromodeoxyuridine
BSA	Bovine Serum Albumin

C2cd2l	C2CD2 Like
Cav1	Caveolin 1
Ccsap	Centriole, Cilia And Spindle Associated Protein
Cd248	CD248 Molecule
CD36	Cluster of differentiation 36
CDKN	Cyclin Dependent Kinase Inhibitor
cDNA	Complementary DNA
cGMP	Cyclic guanosine monophosphate
Chodl	Chondrolectin
Cirbp	Cold Inducible RNA Binding Protein
CNG	Cyclic nucleotide-gated
CNGA	Cyclic nucleotide-gated channel subunit alpha
CNGB	Cyclic nucleotide-gated channel subunit beta
CNGC	Cyclic nucleotide-gated channel
CNS	Central nervous system
CNV	Choroidal neovascularisation
Colla1	Collagen Type I Alpha 1 Chain
Colla1	Collagen Type I Alpha 1 Chain
Colla2	Collagen Type I Alpha 2 Chain
Col27a1	Collagen Type XXVII Alpha 1 Chain
Col5a1	Collagen Type V Alpha 1 Chain
CRX	Cone-Rod Homeobox
Cryo-EM	Cryo-Electron Microscopy
CSNB	Congenital stationary night blindness
CSSI	Centro Servizi Stabulario Interdipartimentale
CTL	Control
CXCL	Chemokine (C-X-C motif) ligand
CYP	Cytochrome P450
Cyp4f13	Cytochrome P450 CYP4F13
Cyr61	Cysteine-rich angiogenic inducer 61

DAPI	4',6-diamidino-2-phenylindole
Dclk1	Doublecortin Like Kinase 1
DHA	Docosahexaenoic acid
DHCR24	24-dehydrocholesterol reductase
DHCR7	7DHC reductase
Dhrs7	Dehydrogenase/Reductase 7
DMAPP	Dimethylallyl pyrophosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmatic reticulum
ERG	Electroretinogram
ERK	Extracellular signal-regulated kinase
Eth-D2	Ethidium Homodimer-2
Etv5	ETS Variant Transcription Factor 5
FBS	Fetal bovine serum
FDPS	Farnesyl Diphosphate Synthase
FPP	Farnesyl pyrophosphate
GC	Guanylyl cyclase
GCAP	Guanylate cyclase-activating protein
GCL	Ganglion cell layer
G	Golgi apparatus
Gdf15	Growth differentiation factor 15
gDNA	Genomic DNA
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GLUT	Glucose transporter
GMP	Guanosine monophosphate

GNAT	G protein subunit alpha transducin
GPCR	G-protein coupled receptor
GPP	Geranyl pyrophosphate
GTP	Guanosine triphosphate
GUCA	Guanylyl cyclase-activating protein
HC	Hydroxycholesterol
HDAC5	Histone Deacetylase 5
HDL	High density lipoprotein
Hist1h2ag	Histone H2A type 1
Hist1h4b	H4 Clustered Histone 2
Hk2	Hexokinase 2
HMGCR	3-Hydroxy-3-Methylglutaryl-CoA Reductase
HMGCS	HMG-CoA synthase
Hsd17b7	Hydroxysteroid 17-Beta Dehydrogenase 7
Idi	Isopentenyl-diphosphate Delta-isomerase
IDL	Intermediate density lipoprotein
IGF	Insulin Like Growth Factor
IL	Interleukin
Il1rl1	Interleukin 1 Receptor Like 1
Il1rn	Interleukin 1 Receptor Antagonist
IMPDH	Inosine monophosphate dehydrogenase
INL	Inner nuclear layer
INSIG	Insulin-induced genes
IPL	Inner plexiform layer
IPP	Isopentenyl pyrophosphate
iPSC	Induced pluripotent stem cells
IRBP	Interphotoreceptor retinol binding protein
IRD	Inherited retinal degeneration
IRE	Inositol-requiring enzyme
IS	Inner segment

JmJd4	Jumonji Domain Containing 4
Kif26b	Kinesin Family Member 26B
Klf2	Kruppel Like Factor 2
KO	Knock-out
LAL	Lysosomal acid lipase
LC-MS	Liquid chromatography–mass spectrometry
LCA	Leber congenital amaurosis
LDL	Low density lipoprotein
LDLR	Low-density lipoprotein receptor
LN521	Laminin-521
Lonrf1	LON Peptidase N-Terminal Domain And Ring Finger 1
LOX	OxLDL receptor
Loxl1	Lysyl Oxidase Like 1
Lss	Lanosterol Synthase
LXR	Liver X receptor
M	Mitochondria
Mant	maintained
Map3k13	Mitogen-Activated Protein Kinase Kinase Kinase 13
Medag	Mesenteric Estrogen Dependent Adipogenesis
MEK	Mitogen-activated protein kinase kinase
MPP	Melamine pyrophosphate
Msmo1	Methylsterol Monooxygenase 1
Mt1	Metallothionein 1A
Mtap	Methylthioadenosine Phosphorylase
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
Murc	Muscle-Restricted Coiled-Coil Protein
MVK	Mevalonate kinase
Mxd1	MAX Dimerization Protein 1
N	Nucleus
NCKX	Na ⁺ /Ca ²⁺ /K ⁺ exchanger

NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NFATC	Nuclear Factor Of Activated T Cells
NFL	Nerve fiber layer
NPC	Niemann-Pick type C protein complex
NRF	Nuclear factor erythroid 2-related factor
NRL	Neural Retina Leucine Zipper
NSDHL	NAD(P) Dependent Steroid Dehydrogenase-Like
OCT	Optical coherence tomography
Ogt	O-Linked N-Acetylglucosamine Transferase
ONL	Outer nuclear layer
OPL	Outer plexiform layer
Optn	Optineurin
OS	Outer segment
OxLDL	Oxidized low-density lipoprotein
PAM	Partitioning Around Medoids
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PEDF	Pigment epithelium-derived factor
PERK	Protein kinase R-like ER protein kinase
PFA	Paraformaldehyde
PKB	Protein kinase B
PKC	Protein kinase C
PKG	cGMP-dependent protein kinase
PMCA	Plasma Membrane Calcium ATPase
PMVK	Phosphomevalonate Kinase
PN	Post natal
Polr3gl	RNA Polymerase III Subunit GL
POSTN	Periostin
Ppa1	Inorganic Pyrophosphatase 1

PRPH	Peripherin
qPCR	Quantitative PCR
RAX	Retina and anterior neural fold homeobox
RD	Retinal degeneration
RD3	Retinal degeneration 3
retGC	Retinal guanylyl cyclase
Rho	Rhodopsin
RLE	Relative Log Expression
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
ROM	Retinal outer segment protein
RP	Retinitis Pigmentosa
RPE	Retinal Pigment Epithelial
RPS26	Ribosomal protein S26
RT-PCR	Real Time-PCR
Runx3	RUNX Family Transcription Factor 3
Sc5d	Sterol-C5-Desaturase
SCAP	Sterol regulatory element-binding proteins cleavage-activating protein
Scd	Stearoyl-CoA Desaturase
SD	Standard deviation
Sesn2	Sestrin 2
Six1	SIX Homeobox 1
Slc25a32	Solute Carrier Family 25 Member 32
Slc39a10	Solute Carrier Family 39 Member 10
SLOS	Smith-Lemli-Opitz syndrome
SQLE	Squalene monooxygenase
SRB	Class B scavenger receptor
SREBF	Sterol regulatory element-binding factor
Tagln	Transgelin
Tgfb2	Transforming Growth Factor Beta 2

Tgfr3	Transforming Growth Factor Beta Receptor 3
TNF	Tumour necrosis factor
Trac	T Cell Receptor Alpha Constant
TSPO	Translocator protein
Txnip	Thioredoxin Interacting Protein
UPR	Unfolded protein response
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
VGCC	Voltage-gated Ca ²⁺ channels
VLDL	Very low density lipoprotein
Wdr36	WD Repeat Domain 36
Wisp1	WNT1-inducible-signaling pathway protein 1
Wnt7b	Wnt Family Member 7B
WT	Wild type
YPEL	Yippee Like
Z-VAD-FMK	Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone
ZAP	Zaprinast
Zc3h6	Zinc Finger CCCH-Type Containing 6
Znrf3	Zinc And Ring Finger 3

ABSTRACT IN ENGLISH

Retinitis Pigmentosa (RP) is a genetic disorder characterized by progressive vision loss due to the degeneration of photoreceptors. More than 100 different genes have been linked to RP, and the high genetic heterogeneity hampers the development of treatments to cure this disabling disease. A more profound elucidation at the molecular level of the mechanisms underlying the progression of the disease is needed for the development of new therapeutic approaches.

This study focused on the study of transcriptional changes during rod photoreceptor degeneration to understand disease mechanisms and find possible therapeutic targets. To gain insights into rod photoreceptor degeneration, we developed a new *in vitro* model based on the 661W cell line that mimics the *PDE6B* gene loss-of-function mutation in RP patients and related animal models. The cell model relies on the treatment with zaprinast, a PDE6 inhibitor, and we compared by RNA-Seq analysis cell exposed to zaprinast *versus* not treated cells. We successfully identified differentially expressed genes in response to PDE6 inhibition, especially related to cholesterol biosynthesis and metabolism. Several gene changes linked to cholesterol biosynthesis and metabolism have been validated by Real-time qPCR in the cell system. Confirmation of the importance of the identified genes in retinal degeneration was performed in relevant animal models of RP.

Finally, because the primary degeneration starts in the rod photoreceptors that are post-mitotic neurons, we developed an *in vitro* differentiation protocol for the new cell line, a fundamental tool for future studies on photoreceptor cell death mechanisms.

In conclusion, we suggest that the cholesterol pathways, characterized in this study, can be evaluated as a new biomarker and as a new target for the development of new therapeutic approaches.

KEYWORDS: Retinitis Pigmentosa; Rod photoreceptor; Cell model; Transcriptome; Differentiation

ABSTRACT IN ITALIAN

La retinite pigmentosa (RP) è una malattia genetica caratterizzata da una perdita progressiva della vista dovuta alla degenerazione dei fotorecettori. Più di 100 geni diversi sono stati correlati alla RP e l'elevata eterogeneità genetica ostacola lo sviluppo di trattamenti per la cura di questa malattia invalidante. Per lo sviluppo di nuovi approcci terapeutici è necessaria un'elucidazione più profonda a livello molecolare dei meccanismi alla base della progressione della malattia.

Questo studio si è concentrato sui cambiamenti trascrizionali che avvengono durante la degenerazione dei bastoncelli al fine di comprendere i meccanismi della malattia e trovare possibili bersagli terapeutici. Per investigare la degenerazione dei bastoncelli, abbiamo sviluppato un nuovo modello *in vitro* basato sulla linea cellulare 661W che simula la mutazione di perdita di funzione del gene *PDE6B* nei pazienti RP e nei modelli animali correlati. Questo modello si basa sul trattamento della linea cellulare con zaprinast, un inibitore della PDE6. Abbiamo poi confrontato tramite analisi RNA-Seq le cellule esposte a zaprinast rispetto a quelle non trattate e sono stati identificati con successo i geni differenzialmente espressi in risposta all'inibizione della PDE6. Tra questi sono emersi, in particolare, geni legati alla pathway del colesterolo. Diversi cambiamenti genici legati alla biosintesi e al metabolismo del colesterolo sono stati convalidati nel sistema cellulare da analisi qPCR. È stata confermata la rilevanza dei geni identificati nella degenerazione retinica tramite analisi in rilevanti modelli animali di RP.

Infine, poiché la degenerazione dei fotorecettori inizia nei bastoncelli, i quali sono neuroni post-mitotici., abbiamo definito un protocollo per il differenziamento della nuova linea cellulare, uno strumento fondamentale per le analisi future sui meccanismi di morte cellulare degli stessi.

In conclusione, lo squilibrio nelle vie del colesterolo caratterizzato da questo studio suggerisce che potrà essere valutato come nuovo biomarcatore o come nuovo bersaglio per lo sviluppo di nuovi approcci terapeutici.

PAROLE CHIAVE: Retinitis Pigmentosa; Bastoncelli; Modello cellulare; Trascrittoma; Differenziamento

1. INTRODUCTION

Seeing helps us to experience different emotions at the same time. The eyes allow us to see the surrounding, the facial expressions of our loved ones, and we can understand another person's thought by simply watching. Eyes let us watch. Sometimes by watching a bird and sometimes whatever we want to watch, they can help us get away from the things that are swirling in our minds. They allow us to create a new world for ourselves just by watching. Eyes enable us to see events, people, behaviors, and landscapes that we can never predict and direct our vision to change. In this way, we can discover life and embark on a journey of self-discovery, and we always experience these things without being aware of our eyes.

The eyes allow us to overcome our ignorance without realizing it. They are instrumental in increasing our intellectuality by our efforts to see what is hidden and unseen in the things we look at. We think that we only see, but in everything we notice by seeing, we adapt without being aware of it. This change in ourselves helps us to understand and accept reality itself as it is, rather than just trying to see what we want to see. Every time we see the bare truth in people, behaviors, and events, the only thing we learn is the word Socrates left us: "All I know is that I know nothing."

The ignorant person is happy. Man is prone to ignorance because ignorance is easy and effortless. For those who do not want to remain ignorant, the effort is the law of life. People are born ignorant, but the goal is to get rid of ignorance. Our eyes help us to overcome our ignorance without realizing it. If there is an event where a critical situation is experienced in life, the person who cannot see is not the dangerous one but is the person who does not want to see; because when you show the person who cannot see the serious situation in some way, the person who cannot see realizes his own mistake and self-deception and changes his thoughts. Even if we show the serious situation to the person who does not want to see it, he does not want to try to see it nor change his thoughts. In order to change, he must be able to admit his own mistake to himself, but he cannot do this and prefers the easy way based on ignorance instead of the difficult one. Seeing helps us overcome the difficult by teaching us the truth and enables us to tame our own ignorance without realizing it. The eyes prevent us from being a victim of illusions and cognitive distortions of our mind. So, eyes give us a purpose, a way that forces us to reach the truth. Shakespeare mentioned this phenomenon in his poems.

All the dark and comfortless.

You can't see your way.

I have no way, and therefore want no eyes.

I stumbled when I saw...

-Shakespeare (King Lear)

1.1. Fundamentals of the Retina

When the light reaches the eye, it first enters through the cornea. The cornea is a clear and transparent tissue in front of the eyeball. The light is refracted by the cornea, and it can continue passing through the pupil located in the center of the iris. Depending on the amount of light, the iris works as a shutter to control the amount of entering light. After passing through the pupil, the light passes through the lens, a specially evolved flexible and transparent part of the eye that can properly focus the light. The eyeball is filled with a gel-like liquid that is called vitreous. The vitreous helps the eye to hold its spherical shape. The light passing through the vitreous reaches the retina. The light is captured in the retina, and the retina converts the light-dependent information to electrical signals to transmit them to the central nervous system.

The retina is a highly specialized and unique neuronal tissue located at the back of the eyeball. The retina works as a biosensor to detect light. The primary function of the retina is the delivery of light-dependent information to the central nervous system thanks to the ability of perceiving, processing, and transmitting light. In humans, the dimension of the retina is approximately 250 mm, and consists of around 100 million cells and presently about 60 types of different cells have been characterized. The retina consists of an unequal organization of the retinal neurons. The retinal neurons are separated in three primary layers. These layers are named depending on cellular functions and the localization of cells bodies (nuclei). These are respectively the outer nuclear layer (ONL), the inner nuclear layer (INL), and the ganglion cell layer (GCL). These nuclear layers are also separated from each other by two plexiform (synaptic) layers. The outer plexiform layer (OPL) is located between ONL and INL, whereas the inner plexiform layer (IPL) is located between INL and GC (Figure 1A) [1, 2].

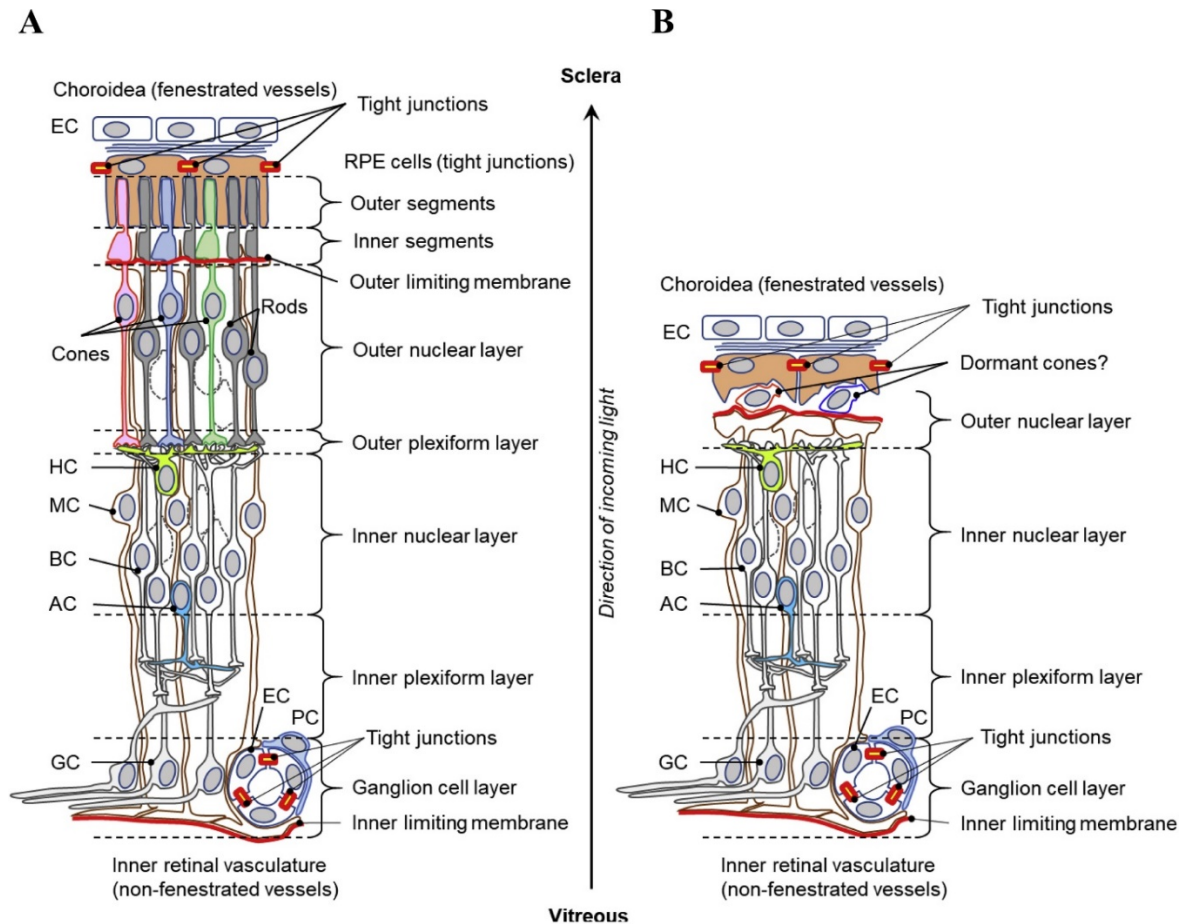


Figure 1. Schematic representation of the healthy and degenerated retina (A) Schematic representation of the major retinal cell types and their organization in the healthy retina. The retina is composed by different specialized layers. From the outermost can be found: the RPE, the outer nuclear layer where the nuclei of rod and cone photoreceptors are located, the inner nuclear layer, and the ganglion cell layer. (B) Representation of the photoreceptor cell loss that occurs at the final stage of retinal degeneration.

AC = amacrine cells; BC = bipolar cells; EC = endothelial cell; GC = ganglion cell; HC = horizontal cells; MC = Müller glial cell; PC = Pericyte; RPE = retinal pigment epithelium. Modified and adapted from [3].

The photoreceptor cells are located in ONL. There are two types of photoreceptor cells called rod photoreceptors and cone photoreceptors. The primary function of the photoreceptors is the absorption of the photons to convert them into electrical signals. The main reason to separate photoreceptors into two types of cells is their absorption capacity in different illumination conditions. Rod photoreceptors are the highly specialized cells for the dim light condition to support night vision in the dark. In contrast, cone photoreceptors are highly specialized for bright light conditions to support daylight vision. Additionally, cone photoreceptors are responsible for high-resolution color vision. The light-dependent information comes with photons to the retina. The photons are perceived by the photoreceptors. The photoreceptors transmit the information to the cells located in the inner nuclear layer (INL) [4].

Bipolar, horizontal, and amacrine cell bodies are placed in the INL. Bipolar and horizontal cells have synaptic junctions with photoreceptors in the OPL. The light stimuli from photoreceptors are received by bipolar cells that transmit them to the GCL. The role of horizontal cells is to integrate the signal coming from multiple photoreceptors forming lateral synapsis. Similarly to the role of horizontal cells in the OPL, amacrine cells in the IPL integrate the signal coming from bipolar cells and modulate the response of ganglion cells [5].

The light stimuli perceived by photoreceptors are transmitted to the bipolar cells and then to ganglion cells. Bipolar cells and ganglion cells form synapses in the IPL and process the visual stimuli. The prominent role of the ganglion cells is to collect all the light-dependent information at a specific time which is conveyed to the central nervous system through the ganglion cell axons that are long neural fibers that form the optic nerve [6].

Additionally, other retinal cells are present in the retina outer and inner parts with roles of protection of the neural retina and of transmission of light-dependent information. Retinal Pigment Epithelial (RPE) cells are in the outer part of the retina facing photoreceptors and constantly cover the photoreceptor outer segments. The RPE cells accomplish several functions such as the recycling of the visual pigment, which combines with the visual pigment protein, called rhodopsin. Moreover, RPE cells are responsible for the phagocytosis and renewal of the photoreceptor outer segments. RPE cells are connected by tight-junctions that help in forming a tight barrier which restricts the molecules that can reach the retina. This limitation protects the neuroretina from the pathogens and toxic molecules that might enter the bloodstream. On account of this, it is called the outer blood-retinal barrier. The glial component in the retina is made of Müller glial cells with cell bodies placed in INL; however, they reach out to all parts of the retina. While they are not involved in the cellular operations to transmit light stimuli, they assist the structural and functional stability of the entire retina, and the apical ends of the Müller glial cells form the outer limiting membrane. The cells are connected with tight-junction as well as adherent-junctions that limit access to the neuroretina. In the inner retina, the vasculature endothelial cells are interlinked by tight-junction. These cells cooperate with pericytes and Müller glial cells to generate the inner blood-retina barrier. An additional barrier is the inner limiting membrane, generated by the basal ends of the Müller glial cells to protect the neuroretina toward the vitreous.

Consequently, RPE cells and Müller glial cells have an essential role in the retinal function, structure, and protection [1, 7].

Overall, the essential structure and function of the human retina provide high-quality vision. While the functionality and lifespan of the cells that compose the retina could be 120 years or more on average, this period can be shortened due to some hereditary diseases. Degeneration of the retina, especially photoreceptors, causes severe impairment of vision and eventually blindness. Most of the retinal degeneration (RD) types of diseases are still untreatable today and affect the population at younger or older ages.

1.2. Inherited Retinal Diseases

Inherited retinal degeneration (IRD) is a group of rare retinal diseases characterized by progressive loss of photoreceptor cells which cause severe vision impairment and eventually leads to complete blindness [8]. Among the IRD disease types, Retinitis Pigmentosa (RP), Stargardt disease, Choroideremia, Achromatopsia are diagnosed in adult-onset or Leber congenital amaurosis (LCA) is diagnosed at early childhood or even infancy. RP is the most common retinal degeneration with an incidence of approximately 1:3,500 in the working population, while LCA has an incidence of 1:8,000 in the population [3, 8].

IRDs are heterogeneous retinopathies that show huge variability genetically and clinically. More than 100 different genes have been identified linked to the disease, and different mutations were found in the same gene (<https://sph.uth.edu/retnet/>). While a mutation in a gene leads to one form of IRD, any change in the location of the mutation in this same gene can cause a different form of IRD; examples are *CRX* or *RP1L1* or *TULP1* genes that can cause RP or LCA (<https://sph.uth.edu/retnet/>). Most of the genes linked to IRDs have important functions in the retina, such as photoreceptor structure, outer segment renewal, phototransduction cascade, retinoid cycle, nucleotide metabolism or can encode for transcription factors and splicing factors [8].

There is close correlation between RP and LCA on the affected biochemical pathways and genes and, indeed, different mutations in the same gene can result in either LCA or RP [9, 10]. In both diseases the outcome can be complete or near-complete loss of the ONL; however, cells in the INL of the retina survive, even if dendrites of bipolar and horizontal cells finally retract [11]. Some LCA phenotypes may affect both rod and cone photoreceptors at the same time [12]. The diagnosis of these different forms of IRD can partially quantify

vision and photoreceptor loss by electroretinogram (ERG) and optical coherence tomography (OCT) [13, 14]. The clinical characteristics of LCA and RP are somewhat indistinguishable and disease progression and clinical characterization show similarity as well as overlap. In fact, LCA and RP are both characterized by the primary loss of rod photoreceptors with LCA showing early dysfunction also of cones. In RP the secondary degeneration of cones usually follows after rod photoreceptor degeneration, even if cone photoreceptors are not genetically affected by the mutations [15]. The biological mechanisms linking the death of rods and cones remain obscure, but it is generally explained as a “bystander effect”, a non-cell-autonomous process comprising multiple factors, responsible for progressively exposing cones to an adverse environment, where inflammation, oxidative stress, deprivation of survival factor prevail. The secondary degeneration of cone photoreceptors has not been explained in detail but can be a noticeable process because in day light we use cones for our vision. Conversely, cone photoreceptors can be affected by mutations in some types of IRD, such as achromatopsia (ACHM). The genes affected by mutations are mainly expressed in cone photoreceptors and lead to the cone photoreceptor primary disfunction and eventually complete degeneration. The clinical features of ACHM are low visual resolution, photophobia, and severe visual impairment from birth [16]. The prevalence of the ACHM is about 1:30,000 in the population. Unlike RP and LCA, in ACHM cones degenerate first, while rods do not degenerate secondarily, and complete vision loss does not occur [17].

1.3. Photoreceptor cells

There are two types of photoreceptors in the outer nuclear layer of the retina (Figure 2). These are named rod and cone photoreceptors depending on their morphologies. The rod and cone photoreceptors are highly specialized sensory neurons that can convert light stimuli into electrical signals interpreted by the central nervous system. In the mammalian retina, rod photoreceptors are more abundant than cone photoreceptors, and their light sensitivity is also higher than the one of cone photoreceptors. The visual pigment in rod photoreceptors is called rhodopsin. The absorbance of a single photon can be perceived by the fully dark-adapted state of rhodopsin and this feature allows scotopic vision in the nocturnal or dim light condition [4].

Cone photoreceptors are distinct from rod photoreceptors functionally and morphologically. They express several kinds of opsins that are visual pigments, and the

different types of cones are based on these pigments varieties. There are three types of cone photoreceptors in the human eye. They are classified based on spectral ranges related to color vision as S, M, and L cone photoreceptors for short-wavelength sensitive cones, middle-wavelength sensitive cones and long-wavelength sensitive cones. Cone photoreceptors can generate a temporally shorter light response, and they are less sensitive to the light than rod photoreceptors. Thanks to all these features, cone photoreceptors provide high-resolution daylight vision, also called photopic vision. The electrical impulse generated as a result of the phototransduction cascade in cone photoreceptors and transmitted to the central nervous system, provides a high-quality visual experience [18].

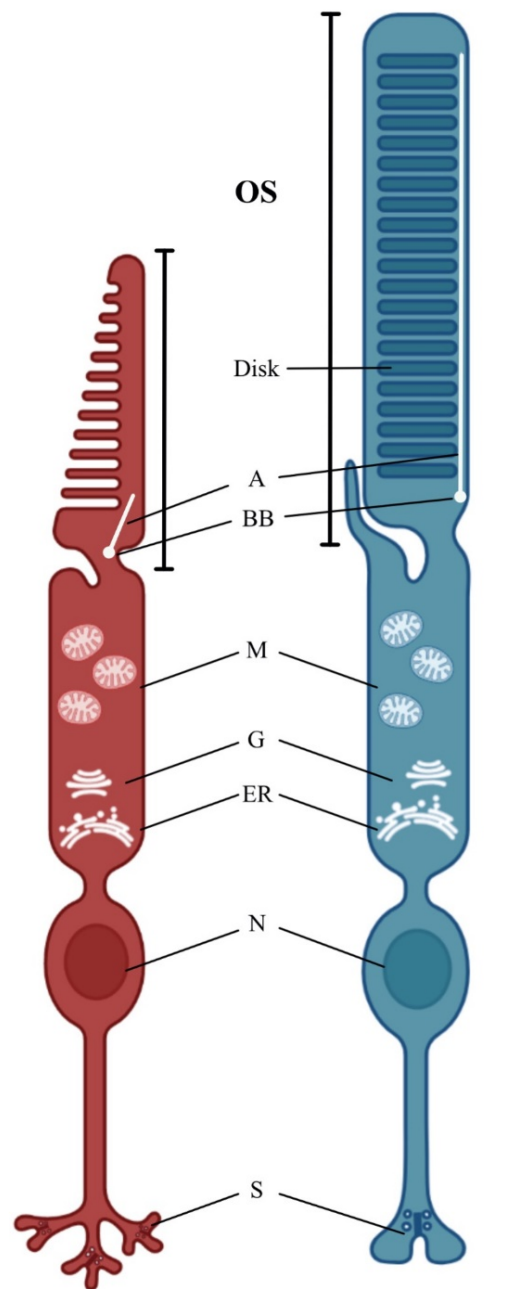


Figure 2. Cone and Rod structure. Photoreceptors are sensory neurons with a highly specialized structure. Both rods (right) and cones (left) have an outer segment (OS) and an inner segment (IS) linked by a connecting cilium. The OS is where the phototransduction cascade takes place, the IS contains all the cellular organelles necessary for cell survival. Finally, there is a synaptic terminal (S) to connect and send the visual signal to interneurons. A, axoneme; BB, basal body; M, mitochondria; G, Golgi apparatus; ER, endoplasmic reticulum; N, nucleus Modified and adapted from <https://biorender.com>

Morphologically, rod and cone photoreceptors are quite elongated and polarized cells (Figure 2). Both cell types contain four subcellular components. These are the outer segment (OS), the inner segment (IS), the nucleus, and the synaptic terminal. The photons are captured by the visual pigments localized in the OS of the photoreceptors and activate the phototransduction cascade. The IS is located immediately proximal to the OS and contains

cellular organelles such as the Golgi apparatus and the Endoplasmic Reticulum (ER) for protein synthesis and mitochondria for energy metabolism. Stimuli, transformed from light to electrochemical signals, are passively transmitted from the photoreceptor axon to the synaptic terminals located in the outer plexiform layer. In the mammalian nervous system, both rod and cone photoreceptor terminal structures are extremely differentiated components. They are characterized by a unit called synaptic ribbon that holds vesicles close to the active zone for the release of the neurotransmitter glutamate in the inner nuclear layer of the retina that contains bipolar and horizontal cells dendrites able to receive the neurotransmitter and activate the impulse [19].

1.3.1. Structure of the Photoreceptor Outer Segment

The OS section of the photoreceptor cells encloses phototransduction components. The fundamentals of this section are to capture the photons and convert them into action potentials at the plasma membrane. Rod and cone photoreceptors have a distinct dimension from each other. A rod photoreceptor is approximately 3 μm in diameter and length ranges between 25 μm and 45 μm . Cone photoreceptors are bigger than rod photoreceptors. However, both rod and cone photoreceptor dimensions depend on several parameters such as animal species, the location in the retina, and the light intensity. Regardless of their size, OS most essential feature is to amplify the signals thanks to the phototransduction cascade [20, 21].

1.3.2. The Phototransduction Cascade in Photoreceptor

The phototransduction cascade, undoubtedly the best studied of all G-protein-coupled signaling pathways, initiates visual transduction. Our understanding of the phototransduction cascade relies on the photoreceptor OS structure. The transduction mechanism is preferentially localized in a specific part of the OS and can be separated into appropriate sections for biochemical studies. The characterization of the transduction cascade benefitted by electrophysiological studies because, single-cell or a group of photoreceptor cells could be detected by patch-clamp and single cell recordings [22]. The phototransduction pathway provides a strong absorption ability. One single-photon can excite rhodopsin by isomerization of the natural ligand, 11-*cis* retinal, which causes a conformational change that, through a biochemical cascade alter the membrane potential that can be measured via

electrodes. Our knowledge has improved thanks to integrating novel technology and electrophysiological research [23].

1.3.2.1. Signal Amplification in Rod Photoreceptor

A distinguishing characteristic of phototransduction in rod photoreceptors is the high sensitivity of this system. Rod photoreceptors can detect even one single photon in the whole dark-adapted stage, translating light into a signal. The phototransduction role is to amplify this signal enormously increasing the one-photon signal. The first step of this amplification starts with rhodopsin, which is composed by the opsin protein in which lysine 296 is bound by a Schiff base to 11-*cis* retinal (G-protein coupled receptor rhodopsin, R). When a photon hits 11-*cis* retinal isomerizing it to all-*trans* retinal, retinal is released and the configuration of opsin protein is changed to R* that can interact with the heterotrimeric G-protein, transducin ($T\alpha$, β , and γ). It results in opening of the switch regions of $T\alpha$, release of GDP and uptake of GTP. This causes the activation of transducin with the complex separated into $T\alpha$ -GTP and $T\beta\gamma$. R* can continue to activate other transducin units during its activation until a mechanism switches it off (83). The role of $T\alpha$ -GTP is to activate the PDE6 enzyme by releasing its inhibitory γ -subunit. Then, activated PDE6 can hydrolyze cGMP molecules to 5'-GMP, resulting in the decrease of the concentration of cGMP in rod photoreceptors causing the closure of the cGMP-gated cation channels. This results in a decrease in the influx of the $Ca^{2+}/Na^{+}/K^{+}$ ions with a hyperpolarization of the plasma membrane [24]. The level of cGMP concentration changes can reflect on the level of the ion channel closure because, if the cGMP decrease is large, it can lead to the closure of all ion channels (Figure 3).

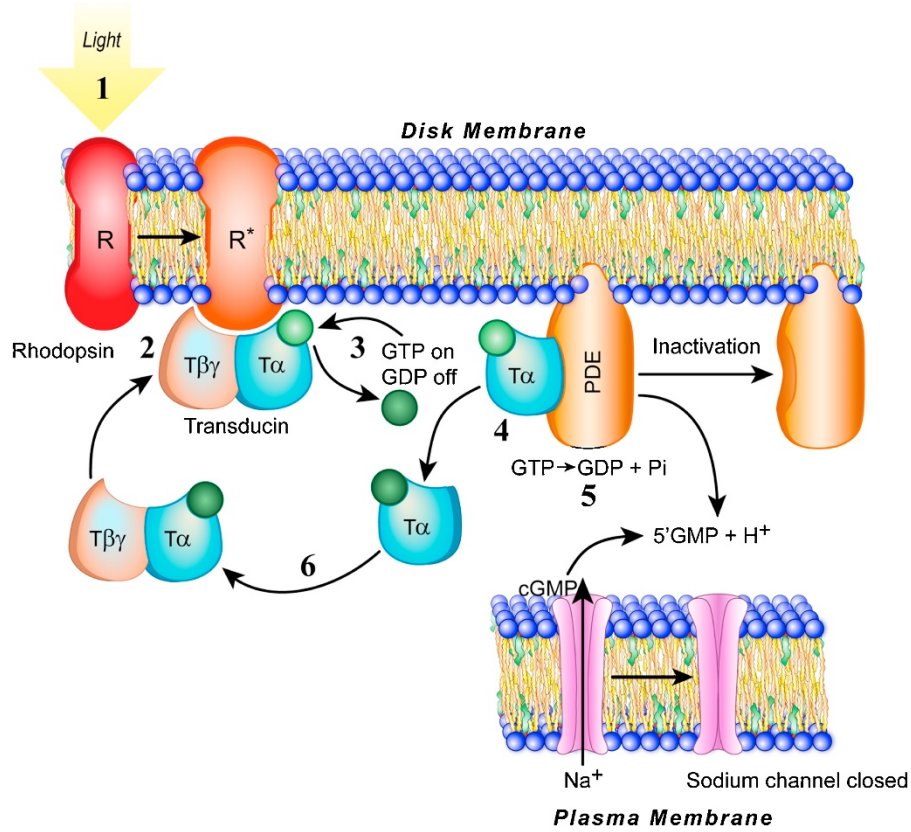


Figure 3. Phototransduction cascade. The phototransduction cascade taking place in the rod and cone outer segments. In rods, light induces a rhodopsin (R) conformational change to an active state (R*) (1). R* interacts with the G-protein heterotrimer transducin (2), promoting the exchange of GDP with GTP (3) and the dissociation of the α -subunit ($T\alpha$). $T\alpha$ binds to and activates phosphodiesterase (PDE) inducing the hydrolyzation of cGMP to 5'GMP and the closure of cyclic nucleotide gated (CNG) channels (5). Thereby, the cell membrane potential hyperpolarizes. Finally, $T\alpha$ hydrolyses GTP to GDP (4) and reassociates to $T\beta\gamma$ (6). Modified and adapted from [25].

The membrane potential of the photoreceptor is depolarized in the darkness. Closing of CNG channels leads to cellular hyperpolarization with decreased release of glutamate at the synapses. In this way, light perception feeling is firstly sent from the photoreceptor to the second-order cells in the retina. In these cells, signals continue to be processed before being sent to the retinal ganglion cells. In order to restore the photoreceptor resting state, the activation processes must be reversed. Initially, catalytically active R^* and PDE^* in the phototransduction cascade must be returned to the resting state and cGMP levels needs to be restored. This process should be fast and repeatable in order to maintain high photoreceptor sensitivity and adaptation to future stimuli (Figure 4) [26, 27].

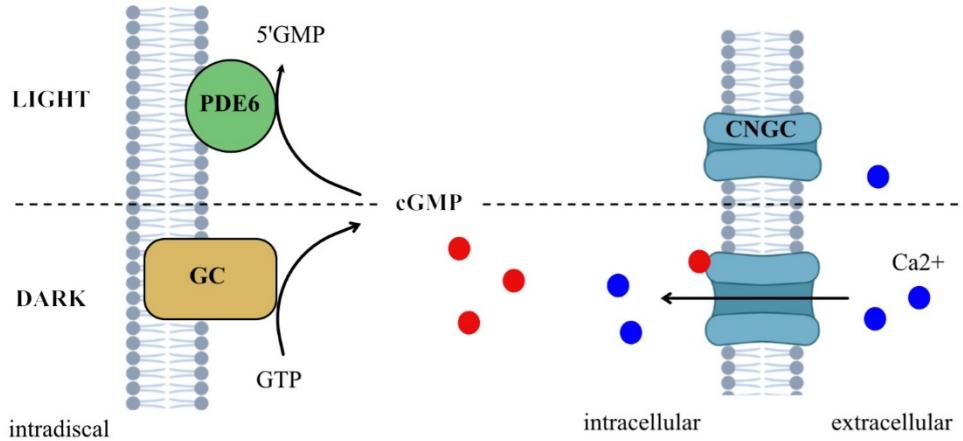


Figure 4. The cGMP in phototransduction. In darkness, guanylyl cyclase (GC) produces cGMP, which opens the cyclic nucleotide-gated channel (CNGC) increasing intracellular Ca^{2+} concentration. In light, phosphodiesterase-6 (PDE6) is activated and hydrolyses cGMP. This causes the closure of CNGC stopping Ca^{2+} influx. Modified and adapted from [3].

1.3.2.2. The Regulation of cGMP and Ca^{2+} Levels in the Phototransduction Cascade

cGMP and Ca^{2+} are second messenger signaling molecules for the phototransduction cascade. Regulation of these molecules has a substantial role in photoreceptor physiology. Dysregulation of cGMP or Ca^{2+} essentially depends on mutations related to the phototransduction cascade and eventually leads to photoreceptor death [28, 29].

In the phototransduction cascade, a high amount of cGMP can exist in dark conditions. retGC synthesizes cGMP in a Ca^{2+} dependent manner. retGC is activated by GCAP to increase the yield of cGMP when the level of Ca^{2+} is low. On the other hand, retGC can be inhibited by GCAP under the high level of Ca^{2+} . This feedback mechanism helps the photoreceptor to regulate the physiological level of the cGMP. In fact, EF protein domains of GCAP, when bound by Ca^{2+} in the dark inhibit retGC, when bound to Mg^{2+} in the light activate retGC. In a Ca^{2+} independent pathway, the activity of retGC is controlled by the RD3 protein [30].

In the dark, a high amount of cGMP causes prototypic effects on the phototransduction targets. It binds and opens the cyclic nucleotide-gated channel (CNGC), which are located in the outer membrane of the photoreceptor OS. When the CNGC is opened, Ca^{2+} and Na^{+} can influx into the OS. Meanwhile, Ca^{2+} ions are continuously extruded via the $\text{Na}^{+}/\text{Ca}^{2+}/\text{K}^{+}$ exchanger (NCKX). When the rhodopsin molecule absorbs a photon, rhodopsin starts sequential activation of G protein transducin and PDE6 enzyme that, as explained above,

hydrolyses cGMP decreasing its levels. When the level of cGMP declines, CNGC gets closed. Subsequently, the cell is hyperpolarized, resulting in the release of glutamate at the photoreceptor synapse. Depending on the closure of CNGC, the level of Ca^{2+} is reduced in the OS, being exchanged with Mg^{2+} at the EF domains of GCAP, which then activates retGC (Figure 5) [31].

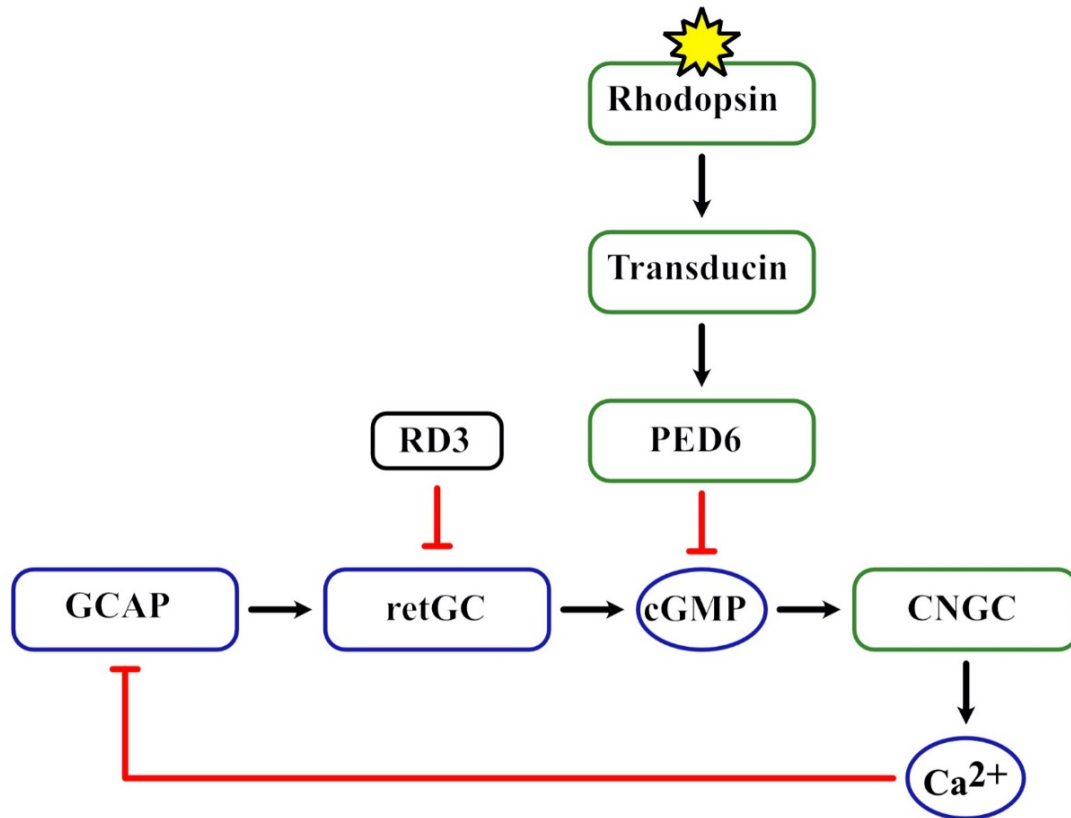


Figure 5. Feedback loop regulates levels of cGMP and Ca^{2+} in the phototransduction cascade. In darkness, the elevated intracellular Ca^{2+} levels prevent guanylyl cyclase-activating protein (GCAP) from activating retinal guanylyl cyclase (retGC). In this way no new cGMP is synthesised. Phototransduction initiates by light activating rhodopsin and consequentially transducin and PDE6. PDE6 rapidly hydrolyzes cGMP leading to cGMP-gated cation channels (CNGC) closure and lowering of intracellular Ca^{2+} concentration. Low Ca^{2+} levels activate retGC, which synthesises new cGMP that re-opens CNGC restoring Ca^{2+} levels. Modified and adapted from [3].

The relationship between photoreceptor degeneration and cGMP was discovered in the early 70s of last century. The researchers established that a high level of cGMP was toxic for cells and causing photoreceptor death [32, 33]. Beginning of the 90s, when the first disease-causing mutation in the PDE6 α and β were discovered, the association of high level of cGMP with photoreceptor degeneration was showed [34]. It became, then, clear that numerous other gene mutations could affect the cGMP and Ca^{2+} levels in exceptionally similar ways. One of the examples is the G86R point-mutation in GCAP1, leading to a

dominant rod and cone photoreceptor degeneration, that increases intracellular cGMP as a result of the regulation of retGC in a Ca^{2+} dependent manner [35]. Another example are mutations in *CNGC* genes resulting in an unbalanced cGMP- Ca^{2+} negative feedback mechanism that causes an enormous increase of cGMP concentration [31]. Moreover, high level of cGMP could be observed in mouse models with a loss of function mutations in the *rd3* gene resulting also in photoreceptor degeneration [30].

1.4. Rod Photoreceptor Cell Death Mechanism

The key point in developing novel treatment strategies for genetically diverse but phenotypically similar diseases is identifying diverse genetic mutations that cause the activation of the same cell death pathway and defining critical matters in the degenerative process that can be therapeutic targets (Figure 6) [8].

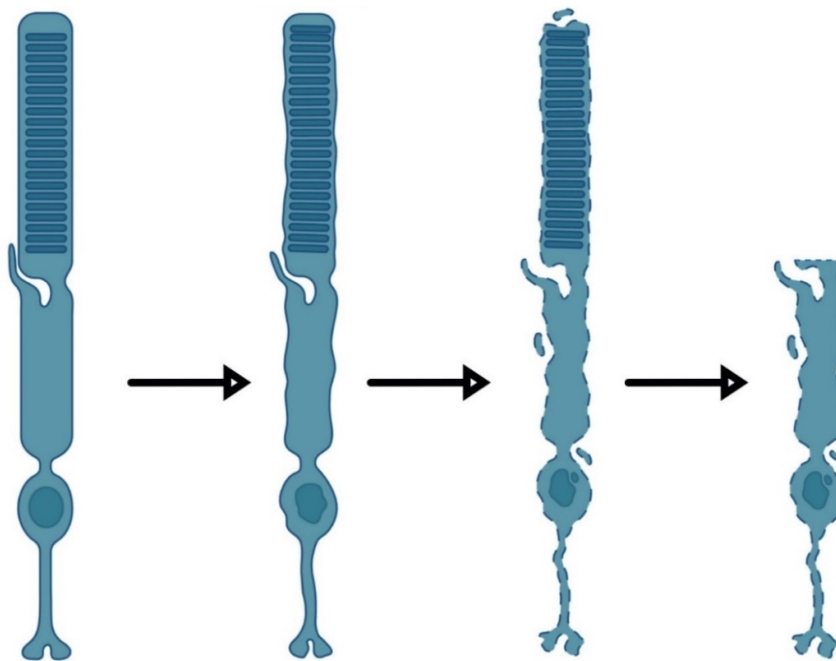


Figure 6. The rod degeneration process Retinitis Pigmentosa is characterised by the progressive loss of rod photoreceptors. Various molecular mechanisms, involving apoptotic and non-apoptotic pathways, trigger rod degeneration leading to a progressive loss of photoreceptor function and death. Modified and adapted from <https://biorender.com>

In photoreceptor degeneration, executioner caspase-dependent apoptotic cell death has been widely debated. The activation of executioner caspases (caspase 3 and caspase 7) was shown in transgenic rats and mice bearing mutations in the *Rhodopsin* (*Rho*) gene [36-38]. In contrast to caspase-dependent apoptotic pathway activation, activation of a caspase-independent pathway was shown in several studies with different models of RP [36, 39].

Knocking out caspase-3 gave only temporary protection to photoreceptors bearing a mutation in the *Pde6b* gene in the *rd1* mouse. This model demonstrated the minor impact of caspase 3 in the photoreceptor cell death pathway [40]. Further studies in which murine IRD models were *in vivo* treated with pan-caspase inhibitors, like Z-VAD-FMK, supported these results. The treatment provided poor neuroprotection [36, 41]. In spite of a caspase-independent cell death, activated BCL2-associated X protein (BAX) was discovered activated in the process of photoreceptor degeneration. The activation of BAX, otherwise, was connected to the efflux from the mitochondria of apoptosis-inducing factor (AIF) and to its translocation to the nucleus. This event was demonstrated in three RP murine models. These RP models were the *rd1* mouse, the knock-out mouse model of *Rho*, and the transgenic mouse model that expresses RHO with the P23H mutation [42]. Many *in vivo* studies suggested that, even though executioner caspases are activated in the IRD, they do not appear to have a driving role in retina degeneration. From this information, it is inferred that the apoptotic cell death term is not suitable for the photoreceptor cell death in IRD. For this reason, "photoreceptor cell death" or "photoreceptor degeneration" terms are preferred by scientists [3, 8].

The endoplasmic reticulum (ER) and mitochondria were also reported to play significant roles in the signaling pathway of photoreceptor cell death. Calcium imbalance with Ca^{2+} ebb and flow in those organelles can be important in photoreceptor cell death. For this reason, the Ca^{2+} flow level is tightly controlled to protect neurons [43]. Photoreceptor cell death studies demonstrated that calcium overload diverges from typical caspase-mediated apoptosis in molecular cell death paths and involves calpains [36, 41, 44].

Calpains are cysteine proteases that are triggered by elevated intracellular calcium. Calpains are heterodimers composed by an 80 kDa proteolytic subunit and a 28 kDa regulatory subunit [45]. In mammalian cells, calpains are classified into two types: typical calpains such as calpain 1, 2, 3, 8, 9, 11, 12, and atypical calpains 5, 6, 7, 8b, 10a, 15. Isoforms of the calpains are involved in many cellular functions, such as signal transduction, apoptosis, differentiation, membrane function, migration, proliferation, muscle fibers formation. Calpains correlates to several events related to neurodegeneration. Calpains are activated during various physiological changes during the disease, such as cytoskeleton fragmentation or changes in cellular membrane permeability. There is a strong correlation between calpain proteolytic activity and cell morphology because increased calpain

activation cause the hydrolysis of cytoskeletal proteins such as paxillin, talin, and vinculin. Moreover, active calpains can increase the permeability in the blood-brain barrier (BBB) through disruption of the tight junctions.

Calpastatin, an endogenous calpain inhibitor, binds to some forms of calpains in the ER. The release of calpastatin, that is regulated by Ca^{2+} binding to the regulatory subunit, triggers the enzymatic activity of calpain proteases [46]. IRD is associated with calpain activation, which stimulates cellular death [47]. Interestingly, a reduction of calpastatin was reported in *rd1* mutant retinas and exposure of the degenerating retina to a synthetic calpastatin peptide could protect photoreceptors from degeneration [48].

Calpains do not have an active role in chromatin condensation. However, these are proteases with a wide range of substrates, including cytoskeleton components, but also some cell death molecules such as AIF and BAX [42, 49]. Calpains can also cleave some caspases, such as caspase 7, 8, and 9, inactivating them, which may be seen as an anti-apoptotic action. However, calpain-1 and calpain-2 have been shown acting primarily as a promoter of cell death because calpain-1 can activate AIF and calpain-2 can cleave and activate pro-caspase-12 [42]. AIF is a flavoprotein found in the intermembrane space of mitochondria. Calpain 1 cleaves AIF favoring its release from mitochondria through a BAX-formed pore in the absence of cytochrome c release, an otherwise mechanism linked to apoptosis [50, 51]. AIF exits from BAX-formed pores in the mitochondria and translocate to the nucleus recruiting the MIF (Macrophage migration Inhibitory Factor) endonuclease to promote chromatin fragmentation [42, 52, 53]. Activation of AIF and its translocation to the nucleus have been found in numerous neural degeneration models, particularly retinal degeneration [41, 54-58]. AIF was also shown to interact with Cyclophilin A which participates in chromatin fragmentation leading to cell death [52, 59].

Calcium overloads induced by dysregulation of many photoreceptor elements can result in calpain activation. In the *rd1* mouse model, the mutated *Pde6b* gene leads to the lack of PDE6 enzymatic activity. In the phototransduction pathway, PDE6 is an essential enzyme and cGMP levels increase when PDE6 function is impaired [32, 60]. The relationship between increased intracellular cGMP and photoreceptor cell death has been well established, and it at least partially explains photoreceptor cell death induced by some IRD-linked mutations [3]. cGMP binds to and keeps open the cGMP-gated channels in the dark in healthy photoreceptors, and this event allows cation entry like Ca^{2+} . The feed-back

loop via GCAP limit cGMP synthesis in these conditions. Therefore, increased cGMP caused by inactive PDE6 ends up increasing the intracellular calcium levels [41]. In addition, elevated cGMP can induce cell death by cGMP-dependent protein kinase enzyme activity [60, 61].

High intracellular Ca^{2+} can also be triggered by mutations causing misfolding of the RHO protein [36]. Dominant mutations in RHO result in protein misfolding and accumulation in the ER. These mutations account for 20-25% of dominant RP forms and about 8-10% of all RP forms [62, 63]. RHO dominant mutations have been investigated for several years, and a variety of mouse models, such as transgenic or knock-in mice, are available. Activation of ER-resident sensors, like IRE (inositol-requiring enzyme 1), ATF6 (activating transcription factor-6), and PERK (protein kinase R-like ER protein kinase), has been reported. On the other hand, their roles in the unfolded protein response (UPR) to initiate protective mechanism or in the cell death mechanism relating in tER-stress are not fully characterized [64-67]. In a transgenic mouse model, mutant RHO was detected accumulated in the ER and this was associated with ER-stress sensor activation to initiate cell death mechanism [37, 58]. In contrast, in the recessive *Rho* knock-out mice no ER-stress could be detected, although a significant Ca^{+2} increase was reported [58]. This observation suggested that the degenerating phenotype caused by mutations in RHO is a dominant phenomenon and not due to haploinsufficiency [58]. Surprisingly, in the knock-in P23H rhodopsin mouse model, which is a model bearing mutant *Rho* in the endogenous *Rho locus* without overexpression, as otherwise found in transgenic mice, activation of the ER sensors, such as phosphorylated IRE1, PERK could be detected but it was demonstrated that it is mostly linked to the UPR response important for degradation of misfolded mutant proteins. Thus, UPR is a compensatory event mostly not associated with cell death [65, 68]. This hypothesis was supported by the evidence that PERK activation induced phosphorylation of nuclear factor erythroid 2-related factor 2 transcription factor (NRF2), an antioxidant modulator which probably plays a protective response in photoreceptor cell death [68]. In fact, PERK inhibition was found to be harmful to the degenerating retina, indicating that PERK maintains the UPR and is a protective response in retinal degeneration [36, 64]. These findings are particularly important for IRD research, because the *Rho*^{P23H/+} knock-in animal mimics photoreceptor cell degeneration, such as gradual degeneration beginning on the ventral side of the retina, in a manner similar to that seen in RP patients bearing the P23H mutation in RHO [69].

To summarize, despite the fact that mutations in the *PDE6B* and *RHO* genes cause either recessive or dominant forms of RP, high intracellular levels of cGMP and Ca^{2+} as well as activation of calpains were identified as key actors in photoreceptor cell degeneration.

1.4.1. The Role of Ca^{2+} and CNGC in Rod Photoreceptor Degeneration

The intracellular Ca^{2+} ions have a major role in photoreceptor functionality but also in their degeneration. Regulation of intracellular Ca^{2+} influx controlled by CNGC has been studied during the last decades as a therapeutic approach for RP. Based on the notion that extreme activation of CNGC causes over influx of Ca^{2+} ions leading to photoreceptor death, some researchers proposed to use a CNGC inhibitor to extend the lifetime of the diseased cells. To this purpose, the complex regulation of intracellular Ca^{2+} levels in photoreceptors needs to be strictly understood.

There are two fundamental Ca^{2+} influx source origins in photoreceptors. The first one is CNGC located in the outer segment, and the second one is the voltage-gated Ca^{2+} channels (VGCC) located at the level of the photoreceptor synapse. A study showed that the registered drug for hypertension, named D-*cis* diltiazem, could be administered to *rdl* mutant mice to hinder rod photoreceptor degeneration preserving the function of the cone photoreceptors [70]. Nevertheless, subsequent studies could not reproduce this result and showed that D-*cis* diltiazem is not effective to rescue of the photoreceptors from cell death as it was proposed [71]. Commercial diltiazem products include two types of diltiazem that are D-*cis* diltiazem and L-*cis* diltiazem. Interestingly, these two enantiomers act on different targets. While L-*cis* diltiazem targets CNGC, D-*cis*-diltiazem targets VGCC. Frasson and colleagues otherwise reported the effect of D-*cis* diltiazem attributing it to a block of CNGC.

Further studies supported the idea that CNGC opening contribute to over influx of Ca^{2+} . A knock-out model to inactivate the rod VGCC was generated in the *rdl* mice to test whether absence of VGCC could rescue the photoreceptor degeneration phenotype caused by mutation of the *Pde6b* gene. There was no rescue effect on photoreceptor degeneration [72]. On the other hand, when the CNGC was inactivated by gene knock-out in the *rdl* mouse, significant reduction in rod and cone photoreceptor cell death was observed [73]. These studies essentially showed that inactivating CNGC could be a therapeutic approach possibly because the calcium influx could be restrained. However, genetic targeting of a specific isoform of CNGC could cause a significant problem in photoreceptors. A recessive

form of RP is in fact caused by mutations in the genes that encode the CNGC subunits (<https://sph.uth.edu/retnet/>). Based on the data that lack of CNGC in rods can protect rod photoreceptors but that a complete loss of function blocks phototransduction and leads to degeneration, search for molecules that can only partially regulate these channels holds interesting potentials as a therapeutic approach. Unfortunately, pharmaceutical products that are specific for CNGC isoforms have not been developed yet.

The heterotetramer structure of CNGC has an α and a β subunit and the channels function is similar in both rod and cone photoreceptors. Different sets of genes encode the different subunits of the CNGC in rods and cones. In rod photoreceptors, the channel subunits, encoded by *CNGA1* and *CNGB1* genes, arrange in a stoichiometric 3:1 ratio. *CNGA3* and *CNGB3* genes encode the channels of the cone photoreceptor and the corresponding protein ratio is similar to the one in rod photoreceptors (*i.e.* 3:1) [74]. The idea of selectively knocking out only the CNGB1 subunit could represent a new therapy. However, limitations depend on the structure of the CNGB1 gene that is encoded by 33 exons with isoforms derived by alternative splicing and this same gene also encodes the glutamic acid-rich protein (GARP). GARP has a critical role in photoreceptor function and survival because of its interaction with peripherin-2. Peripherin-2 is a structural protein in the region of contacts of photoreceptor OS disks to the plasma membrane. While the inhibition of CNGB1 is considered a molecular approach, inactivation of the gene may affect GARP as well. Moreover, targeting CNGCs should be carefully considered because of unbalanced Ca^{2+} caused by lack of CNGC may, through the feedback loop mediated by GCAP, lead to increased cGMP production.

If Ca^{2+} ion influx is mainly through CNGC and VGCC, at the photoreceptor plasma membrane there are pumps to extrude the ion from photoreceptors, such as the Ca^{2+} ATPase (PMCA) pumps. A recent study showed that the neurotrophic factor pigment epithelium-derived factor (PEDF), by reinforcing the activity of PMCA, protects photoreceptors from cell death. PEDF could, in fact, lower intracellular Ca^{2+} level and, consequently, restrain calpain activation [66]. Based on these data PEDF could be evaluated for neuroprotective treatments of the degenerating retina.

1.4.2. Cyclic GMP–dependent protein kinase Activation in Photoreceptor Cells

In retinas of PDE6 mutant mouse models, three times higher levels of cGMP have been observed compared to healthy retinas. In rod photoreceptors, a tremendous accumulation of cGMP has been reported not only upon mutation in PDE6 but also in murine models bearing mutations in other genes, such as mutants in CNGB, in rhodopsin, and in guanylyl cyclase [75]. cGMP not only interacts and opens CNGB but can also bind the regulatory domain and activate the catalytic domain of PKG enzymes [76]. Activated PKG can phosphorylate several proteins, and, among those, cytoskeleton proteins like Vasodilator-stimulated phosphoprotein (VASP), with several effects on neuronal, cardiovascular, and intestinal physiology [77]. There are three PKG isoforms encoded by two genes: *PRKG1* encodes for two isoforms (PKG1 α and PKG1 β) derived from alternative first exons [78]. *PRKG2* encodes for PKG2 [79]. All PKG isoforms are expressed in the retina [80]. Activation of PKGs during retinal degeneration was correlated to photoreceptor cell death in the *rd1* and in the *rd2* mutant mice [61]. PKG activity was significantly increased also in *Cnga3* mutant retinas [81].

The apoptotic effect of PKG activation on several cancer types has been studied [82, 83]. These studies showed that specific activation of PKG2 with novel cGMP analogues can limit melanoma cancer growth and invasion [84, 85]. Nevertheless, the mechanisms of the downstream signaling pathways of PKG1 and PKG2 have not been characterized yet. Targeting PKGs is a therapeutic option that needs to be evaluated despite the limited information that we have on the cell death mechanisms activated by PKGs.

1.5. Genes Linked to cGMP Dysfunction Related to Photoreceptor Degeneration

RD is described with a wide range of genetic heterogeneity. Identified mutant genes are more than 270 (<https://sph.uth.edu/retnet>; information up to 2019). For this reason, enlightening of the photoreceptor degenerative mechanisms and development of treatment strategies are difficult tasks. Mutations in different genes can be recessive, dominant, and X-linked and may result in gain-of-function or loss-of-function effects. The same gene can be affected by either type of mutations generating a more complex disease phenotype [86]. One example is related to mutations in the *GUCY2D* gene, which codes for retinal guanylyl cyclase (retGC), the enzyme synthesizing the second messenger molecule cyclic guanosine-3'-5'-monophosphate (cGMP) in photoreceptor outer segments. Above 100 mutations have

been identified in the *GUCY2D* gene [10]. When the mutation causes loss-of-function, the photoreceptor stops producing cGMP. Therefore, photoreceptors die due to low cGMP levels [87]. However, many mutations in the *GUCY2D* gene induce gain-of-function effects that result in permissive activation of the enzyme and photoreceptors generate an excessive amount of cGMP [88, 89]. The *GUCA1A* and *GUCA1B* genes can also be mutated in different ways [90, 91]. These genes generate the guanylyl cyclase-activating protein (GCAP) which regulates retGC activity in a Ca^{2+} -dependent manner in rods and cones [28]. Generally, loss-of-function mutations lessen Ca^{2+} binding to GCAP and result in cGMP overproduction [35, 92]. As a result, retGC gain-of-function mutations and loss-of-function mutations on GCAP have similar effects, that are high-level of cGMP in photoreceptors. Another example of the diversity of mutation effects in RD genes refers to photoreceptor phosphodiesterase-6 (PDE6) [93]. Rod PDE6 is a heterotetramer composed by two catalytic and two inhibitory subunits. The catalytic subunits are encoded by *PDE6A* and *PDE6B* genes encoding the α and β subunits. The *PDE6G* gene encodes the inhibitory γ subunits. On the other hand, the cone PDE6 enzyme is a heterotetramer with two catalytic α subunits and two inhibitory γ subunits. *PDE6C* gene encodes the α subunits, and the *PDE6H* gene encodes the γ subunits. Many PDE6 mutations, especially on the *PDE6A*, *PDE6B* and *PDE6G* genes, cause RP [94-96] or on the *PDE6C* and *PDE6H* cause ACHM [97-99]. Loss-of-function mutations in the PDE6 enzymatic subunits result in an abnormally high level of cGMP level. The photoreceptor cells are affected by this cGMP level and end up with cell demise [32, 61, 100, 101]. In some clinical cases, rod function is lost without cell death as found with some mutations in the *PDE6A* gene. These clinical conditions are congenital stationary night blindness (CSNB) [102]. These are just some examples of the complex gene mutation scenario in RD linked to cGMP metabolism disruption. These examples reveal that specific mutations in RD linked genes require careful examination to push forward the development of therapeutic strategies.

Manipulation in cGMP-signaling may offer a novel treatment strategy suitable to many distinct RD-causing mutations. Previous studies demonstrated that therapies based in appropriate drug-delivering of pharmacologically active compounds can be possible in RD [61, 103]. In order to translate these new therapeutic opportunities to the clinic, some questions need to be address, such as: i) the number of RD genes and mutations that can be targeted by compounds able to modulate the cGMP pathway; ii) the delivery route of such a compound that will need to be supplied chronically to patients; iii) the number of patients

that can be susceptible to such treatment, an important information for the development of the clinical trial. As previously stated (see chapter 1.5), in RD, mutations in PDE6 subunits inhibit hydrolysis of cGMP and cause an enormous accumulation of the cyclic nucleotide in photoreceptors [34, 95, 99, 101, 104, 105]. Additionally, mutations in aryl hydrocarbon receptor-interacting protein-like gene (*AIPL1*) [12] prevent dimerization of the PDE6 enzyme [106] impairing its function and result, as well, in excess cGMP inside the photoreceptor cell. Transducin, a protein encoded by the *GNAT1* gene in rods and *GNAT2* in cones, activates PDE6 during the phototransduction process, which explains why some mutations in these genes result in inadequate cGMP hydrolysis [107, 108].

Although inadequate cGMP hydrolysis results in high cGMP levels in photoreceptors, abundant synthesis may also have this impact. As a result, gain-of-function mutations affecting retGC, a protein transcribed by the *GUCY2D* gene [109], and also GCAP, which is encoded by the *GUCA1A* and *GUCA1B* genes in rods and cones [35, 91], will lead to excess cGMP synthesis. Finally, since the RD3 protein inhibits retGC, loss-of-function mutations in the *RD3* gene induce abnormal cGMP synthesis and photoreceptor degeneration [30].

Mutations in the CNGC channel, encoded by in the genes *CNGA1*, *CNGA3*, *CNGB1*, and *CNGB3* [75, 110-113], are related to an excess cGMP in photoreceptors, probably due to the absence of negative feedback on retGC based on Ca^{2+} inflow in the outer segment which is impaired due to mutations in the channel that regulate cation influx in photoreceptors [31].

Remarkably, high cGMP levels have been detected in animal models with mutations in numerous additional genes that are not connected directly to cGMP production or hydrolysis. For example, many diverse mutations in the rhodopsin (*RHO*) gene, varying from a complete knock-out to the dominant P23H point mutation or S334ter truncation, exhibited increased cGMP levels in photoreceptors [75]. However, we cannot deduce that cGMP buildup in photoreceptors occurs in all of the 150 *RHO* mutations [62] identified today.

Mutations in the genes related to the outer segment structure protein, peripherin (*PRPH2*), also caused accumulation of cGMP [61, 75]. Ultimately, loss of function of *PRPH2* may contribute to excessive and dysregulated production of outer segment enzymes. Peripherin and retinal outer segment protein (*ROM1*) bind to the photoreceptor disc in the

outer membrane part of the OS [9]. Outer segment disorganization and shortening can occur with *Prph2* and *Rom1* loss-of-function in mouse models which ends up with photoreceptor degeneration [114]. It is expected that *ROM1* mutations may be linked to excess cGMP levels due to the similar role to *PRPH2*. Identifying the impact of such gene changes on photoreceptor cGMP might indirectly help researchers to deduce the circumstances that connect outer segment stability to cGMP metabolism.

The inosine monophosphate dehydrogenase-1 (*IMPDH1*) gene mutation may also cause excess cGMP. The *IMPDH1* enzyme catalyzes GTP in a rate-limiting step and this is the substrate used by *retGC* for the synthesis of cGMP. Interestingly, the mutations in *IMPDH1* were not found to lower its enzymatic activity [115] but could somehow alter GTP synthesis [116]. They may even lead to more GTP production, which, given the relatively high Michaelis constant of *GC* [117], might lead to an increase of cGMP synthesis.

Besides the RD genes described above, for which the link to excess cGMP in photoreceptors has previously been established or seems to be highly plausible, mutations in other photoreceptor-specific genes will possibly induce unbalanced cGMP. The transcription factors *CRX*, *NRL*, and *NR2E3*, that control the expression of genes involved in phototransduction [81, 118], might be potential candidate for such a connection. When these genes are defective by mutations, phototransduction activity may be unpaired. Similarly, phototransduction protein trafficking genes, such as *REEP6*, that is necessary for delivery of *retGC* to the OS of photoreceptor cells [119]. It is still unclear whether mutant *REEP* may cause RD because of excessive cGMP in photoreceptors.

On the other hand, an excessive amount of cGMP may also be linked to Stargardt disease caused by mutations in the *ABCA4* gene [120]. The activity of *ABCA4* is required to transport all-*trans*-retinal from the photoreceptor disks to RPE cells for recycling [121, 122]. Consequently, *ABCA4* mutations are linked to early abnormalities in the retinal electroretinographic responses [123, 124]. Previous studies suggested that inadequate retinal recycling reduces the efficiency of phototransduction. It is possible that this event is associated to a decrease cGMP hydrolysis and cause cGMP accumulation in a time-dependent manner.

To sum up, many RD genes are linked, or possibly can be linked, to an excessive amount of the cGMP. These are RD genes are involved in cGMP production, hydrolysis, or

encoding for CNGCs. Even though many disease gene expressivity vary according to patients' cohorts, ethnic and the place of living, the RP linked with excess cGMP can be estimated to up to 30%.

1.6. Sterols and Retina

Sterols denote a broad class of plasma membrane lipids found in all eukaryotic cells [125]. Cholesterol appears to be the most abundant sterol detected in mammalian cells and tissues. The maintenance of correct cholesterol levels is critical for proper cellular function and survival, with the balance of endogenous de novo synthesis, external uptake, and sterol efflux playing a significant role. Cholesterol homeostasis mechanisms have been widely studied due to their function in biological processes and their alteration in numerous clinical conditions, including Alzheimer's disease [126], Parkinson's disease [127], cardiovascular disease [128], and age-related macular degeneration (AMD) [129]. In this section the mechanisms of cholesterol homeostasis in the neural retina will be described, as well as the pathogenic mechanisms behind various ocular disorders due to disrupted cholesterol homeostasis.

The retina is a unique tissue of the central nervous system (CNS). Within the CNS, cholesterol is predominantly found in nerve myelin and the plasma membrane of neuronal and non-neuronal cells. De novo synthesis of the sterols is essential in the brain, as the uptake of lipoproteins is restricted by the blood-brain barrier (BBB) [130]. The retina exhibits more a complex sterol homeostasis when compared to other tissues in the human body; the reasons for this could be the variety of the sterol sources, as well as de novo synthesis. The retina, in fact, carries out uptake of cholesterol in addition to sterol biosynthesis. Cholesterol and sterol precursors may be enzymatically and non-enzymatically oxidized and the pro-oxidative environment within the retina leads to the release of toxic oxysterol derivatives, with potentially damaging effects to the cells [131, 132]. The progression of Parkinson's disease, Alzheimer's disease, AMD, and other retinopathies may be affected by sterols and sterol metabolites [133].

The unique structure of photoreceptor cells provides some anatomical advantages in terms of studying cholesterol homeostasis. The IS and OS of the photoreceptor cells are specialized for the different intracellular activities; the IS of photoreceptor cells acts as a hub

for biosynthesis, as explained in chapter 1.3 whereas the OS accommodates membranes for the phototransduction cascade.

The OS membrane consists of approximately equal amounts of proteins and lipids. Glycerophospholipids are the most dominant lipid in the membrane, making up approximately 80–85% of total lipids. The level of cholesterol in the OS membranes is well-defined and changes when new membranes are produced by the photoreceptor cells. The main types of proteins within the plasma membrane are visual pigments, constituting approximately 90% of overall components. Because the OS disc membranes of the photoreceptors are daily shredded by phagocytosis by the RPE cells [134-136], in order to compensate for this loss and maintain its structure, the retina utilizes *de novo* cholesterol synthesis and uptake of cholesterol from choroidal blood vessels and from the outer and inner retinal veins [25, 137]. Furthermore, the outer and inner plexiform layers containing many synaptic connections and synaptic vesicles, also contribute to the transport of cholesterol [138].

Below, I will describe overall cholesterol homeostasis in the retina, depending on *de novo* synthesis, uptake, and efflux. I will also specifically discuss the role of cholesterol in photoreceptor cells, as well as the structural and functional effects of cholesterol in the OS of the rod photoreceptors, visual proteins, and phototransduction cascade. In addition, cholesterol-related macular degenerations are summarized.

1.7. Cholesterol Homeostasis in the Retina

Cholesterol homeostasis is a well-organized system dependent on input and output of cholesterol in the retina. Retinal homeostasis utilizes several ways of regulating intracellular cholesterol levels. Cholesterol can be uptaken directly from the blood through the LDLR (low-density lipoprotein receptor) localized on the basolateral side of RPE cells facing the choroid [139, 140]. Locally synthesized cholesterol also contributes to cholesterol input in the retina, with one study showing that 72% of cholesterol in the mouse retina originates from *in situ* biosynthesis [141]. Several pathways to export excess cholesterol also play crucial roles in the retina, *e.g.*, the reverse cholesterol transport pathway and metabolism of excess cholesterol to oxysterol forms catalyzed by CYP enzymes. The reverse transport pathway is based on cholesterol efflux in which HDL and apolipoproteins act as acceptors of excess cholesterol, while ABCA1 (ATP-binding cassette subfamily A member 1),

ABCG1 (ATP-binding cassette subfamily G member 1), and ABCG4 acts as transporters. Cholesterol efflux via this transport system occurs in RPE cells and choroidal endothelial cells [142-144].

Several factors regulate retinal cholesterol homeostasis including SCAP (sterol regulatory element-binding proteins cleavage-activating protein), SREBF2 (sterol regulatory element-binding factor 2), LXR- α /NR1H3 and LXR- β /NR1H (liver X receptor α and β), and INSIGs (insulin-induced genes) [145]. SREBF2 is master regulator of cholesterol biosynthesis. When intracellular levels of cholesterol decrease, SCAP induces translocation of SREBF2 from the ER to the Golgi apparatus, where SREBF2 is cleaved by two proteases, leading to activation of the N-terminal domain that functions as a transcription factor. SREBF2 upregulates genes involved in uptake and biosynthesis of cholesterol. In contrast, when the level of cholesterol increases in the cell, the interaction of cholesterol with SCAP inhibits contact of SCAP with INSIGs, resulting in trapping of SCAP in the ER, and decreased activation of SREBF2 [146-148]. Additionally, cholesterol biosynthesis can be inhibited by oxysterols. When oxysterols bind to INSIGs, SREBF2 processing is blocked. Moreover, oxysterols can activate the LXR transcription factors that positively regulates cholesterol homeostasis by increasing cholesterol efflux [149-152].

1.8. Cholesterol Biosynthesis, Uptake and Efflux

The cholesterol biosynthesis pathway (Figure 7) includes a rate-limiting step, the mevalonate pathway, which is catalyzed by the HMG-CoA reductase enzyme (HMGCR). In later steps, the pathway enters the post-squalene phase, in which squalene-2,3-epoxidase (squalene monooxygenase, SQLE) is responsible for regulation. The linear isoprenoid products of the mevalonate pathway play a role in the prenylation of proteins (farnesyl diphosphate, geranylgeranyl diphosphate), N-glycosylation of proteins (dolichol and its phosphorylated derivatives), and sterol (squalene) synthesis [153-155]. Cycling of squalene is achieved by the Kandutsch-Russell and Bloch pathways, resulting in the sterol intermediates lathosterol, 7-dehydrocholesterol (7DHC), lanosterol, 7-dehydrodesmosterol, and desmosterol; these sterol intermediates are then converted to cholesterol by 7DHC reductase (DHCR7) and 24-dehydrocholesterol reductase (DHCR24). Intermediate sterols and the metabolic pathway of these sterols are critical for organisms. For this reason, mutations in any of these enzymes can result in recessive and mostly fatal pathologies [156, 157]. Examples of inherited diseases are: Smith-Lemli-Opitz syndrome (SLOS), caused by

deficiency in DHCR7; lathosterolosis, which results from a deficiency in sterol-C5-desaturase enzyme; desmosterolosis, caused by a deficiency in DHCR24; and deficiency in mevalonate kinase (MVK).

Besides *de novo* synthesis of cholesterol, cholesteryl esters can be imported into the cell by receptor-mediated endocytosis of blood-borne, liver-derived lipoproteins (IDL, VLDL, LDL; 21, 22). Free cholesterol is produced by lysosomal acid lipase (LAL) in the lumen of the lysosome and then transported to the ER by the Niemann-Pick type C protein complex I and II (NPC-I/NPC-II). Mutations in NPC-I/NPC-II cause accumulation of free cholesterol in lysosomes by interfering with its transport and result in the Niemann-Pick's disease [158-160]. Conversely, cholesteryl esters can be selectively uptaken from HDL particles via class B scavenger receptor I (SRB-I). The cell endocytoses OxLDL through the cluster of differentiation 36 (CD36) receptor and OxLDL receptor (LOX-1).

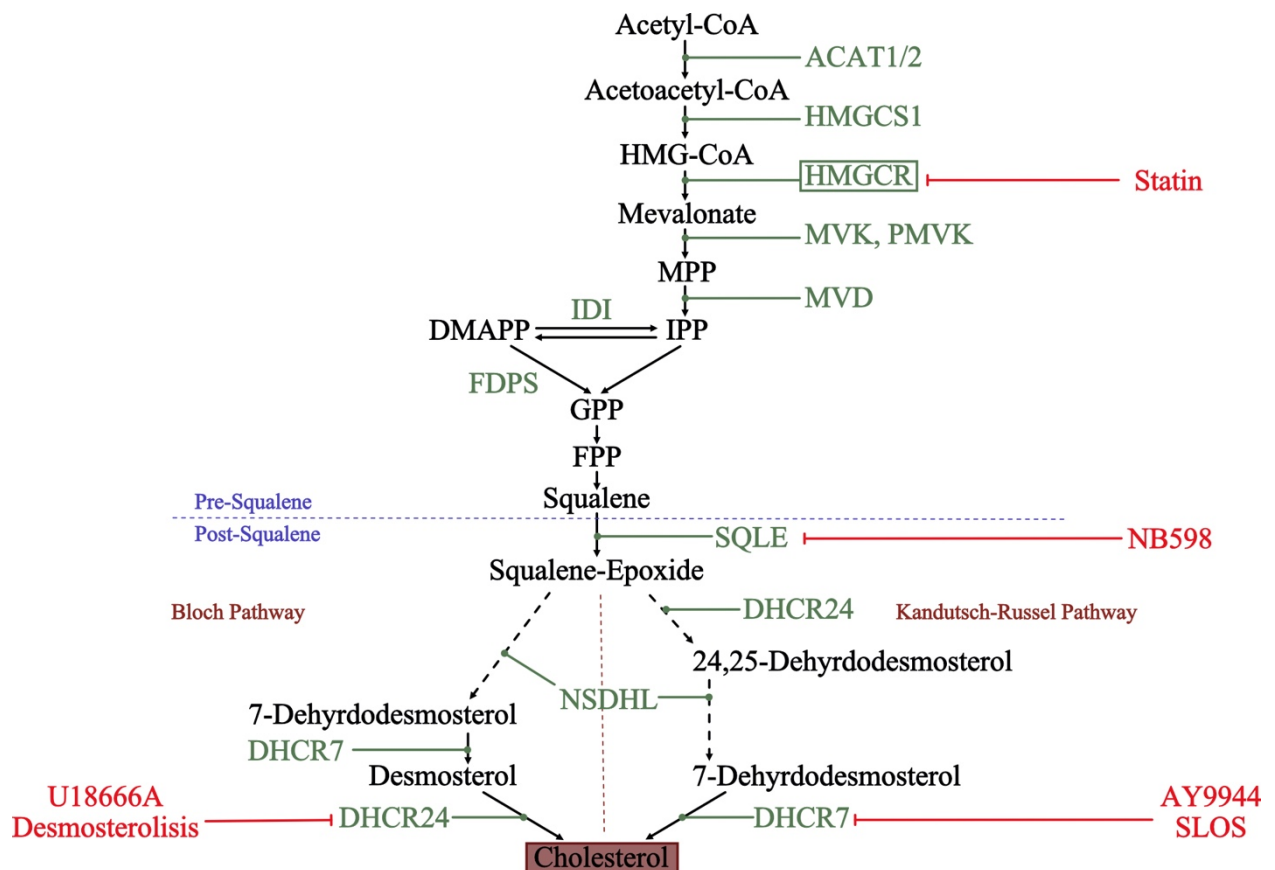


Figure 7. Schematic representation of the mevalonate pathway. Schematic representation of the mevalonate pathway. During this process, Acetyl-CoA is converted by ACAT1 and ACAT2, and subsequently by HMG-CoA synthase 1 (HMGCS1), and HMG-CoA reductase (HMGCR) to mevalonate. HMGCR catalysis is the main rate-limiting step in the pathway and it is inhibited by statins. Starting from Mavelonate, the pre-Squalene portion of the pathway generates a series of acyclic isoprenoid compounds (MPP, IPP, DMAPP, GPP, FPP) and finally Squalene is synthesized. In the post-Squalene portion of the pathway, squalene epoxidase (SQLE; inhibited by NB-598) catalyses epoxidation of squalene to squalene-2,3-epoxide. Following reactions of demethylation, doublebond isomerization and reductions finally lead to the formation of Cholesterol either through the Bloch Pathway or the Kandutsch-Russell Pathway. The last step involves the reduction of the side-chain double bonds in desmosterol or 7-dehydrosdesmosterol and are catalysed by DHCR24 (inhibited by U18666A) or DHCR7 (inhibited by AY9944). Modified and adapted from [138].

ABC transporters are a huge family of physiologically significant molecules that are involved in the efflux of different substrates including peptides, proteins, ions, membrane lipids, and lipid-soluble compounds. Transporters such as ABCA1, ABCG4, and ABCG1 mediate cholesterol efflux from peripheral tissue in the retina to form apolipoprotein A1 (APOA-1)-containing HDL [161, 162]; their role, and the role of APOE-containing LDL particles [163, 164], is critical for this process. In general, deficiency in ABC transporters causes serious chronic diseases associated with structural and functional abnormalities in the eye. For instance, deficiency of ABCA-1 is linked to atherogenesis and neurological disorders such as Alzheimer's disease [165, 166]. Tangier disease, caused by recessive mutations in ABCA1, is characterized by low levels of APOA-1-HDL (hypoalphalipoproteinemia), leading to moderate neuropathy, retinopathy, atherosclerosis,

infiltrates and scarring of the cornea, lipid deficit in the conjunctiva, and development of cataracts [167].

Intracellular LXR agonists and antagonists have a significant modulatory effect on cellular sterol efflux [151, 168], because LXR- α regulates the transcription of genes for sterol efflux, *e.g.* genes encoding ABC transporters [169-171]. Sterols are oxidized or hydroxylated by CYP enzymes (CYP27A1, CYP46A1, CYP11A1, CYP39A1, CYP7A1), resulting in sterol metabolites that act as powerful antagonists of LXR [172, 173]. Cerebrotendinous xanthomatosis, an autosomal recessive disease characterized by chronic diarrhea, neuronal malfunction, early-onset of atherosclerosis, and visual processing impairments such as cataract development, is associated with mutations in a crucial CYP enzyme found in the mitochondria, CYP27A1 [174, 175]. The CYP enzymes generate physiologically significant metabolites by acting on a wide range of substrates, particularly sterols and oxysterols. CYP7A1, an enzyme that transforms 7DHC to 7-oxocholesterol and 7-ketocholesterol (7KChol), also contributes to the pathophysiology of SLOS and cerebrotendinous xanthomatosis [176, 177]. CYP27A1 helps to break down cholesterol to 27-COOH-Chol and 27-OH-Chol, and 7KChol to 27-COOH-7KChol and 27-OH-7KChol through acid hydroxylation [170, 178], increasing the ABCA1/G1-mediated cellular efflux of cholesterol [179, 180]. In conclusion, the dynamic interaction of *de novo* cholesterol synthesis, uptake, and efflux pathways, and the metabolism of cholesterol in the retina are key steps for cholesterol homeostasis and retinopathies can emerge when these key homeostatic mechanisms are disrupted.

1.8.1. Cholesterol Biosynthesis and Uptake in the Retina

Radiolabeling of atoms is a practical and common technique in biochemical studies. Radiolabeling techniques were also applied in early *in vivo* and *in vitro* experiments in animal models to identify cholesterol biosynthesis pathways in the retina. Fliesler and Schroepfer utilized radiolabeling for experiments on the bovine retina [181]. Early *in vitro* studies utilized intact bovine retinas for organ culture, as well as retinal cell homogenates, consisting of microsome and cytosol [181-183]. Sterol production in the vertebrate neural retina was demonstrated by treatment with [^3H]mevalonate. Cholesterol biosynthesis was also observed using radiolabeled carbon intermediates of the mevalonate cascade (isoprenoid acids). Initial *in vivo* studies on cholesterol production used intravitreal injections of [^3H]acetate in rats; acetate incorporation was measured with and without

lovastatin, a HMGCR inhibitor [184, 185]. This experiment showed production of cholesterol within six hours in the neural retina, with very little accumulation of intermediates. Cholesterol production was completely blocked by lovastatin co-injection [184-186]. In addition, radiolabeled squalene accumulation was induced by the squalene 2-3 epoxidase inhibitor, NB-598, which inhibits the post-squalene step of the cholesterol cascade. Other experiments showed similar results with [^3H]farnesol intravitreal injection in rats, demonstrating that [^3H]cholesterol production was not affected by NB-598 [187]. These findings first established the presence of *de novo* sterol synthesis throughout the retina. Nevertheless, this metabolic technique allowed the calculation of exact cholesterol synthesis rates [188-190].

Intravitreal injection of lovastatin causes early functional alterations in the neural retina. The effects of lovastatin were initially attributed to interruption of cholesterol production; however, further research has shown that the reason is improper prenylation of proteins [191]. Similarly, crucial evidence regarding the function of pre-squalene and post-squalene cascades in the rat retina was revealed by the use of pharmacologically targeted HMGCR and SQLE but the function of the mevalonate shunt pathway in the retina has not been characterized yet. Prolonged injection of deuterated water ($[\text{}^2\text{H}]$ water) and subsequent mass spectrometry analysis of the cholesterol isotopomer distribution allowed for *in vivo* assessments of the actual levels of tissue cholesterol production. According to a recent study that used the isotopomer technique, most (>70%) of retinal sterol is synthesized *de novo* [141]. However, accurate estimation of tissue sterol synthesis rates through this technique necessitates careful consideration of many significant elements, including distribution of the molar fraction in the tissue, deuterated sterol enrichment (that is, the average amount of $[\text{}^2\text{H}]$ atoms for every novel synthesized sterol derivatives), experimental confirmation of $[\text{}^2\text{H}]$ cholesterol synthesis in steady-state, and an experimentally specific correction factor to explain each novel cholesterol production [192-195]. According to a recent study that used the isotopomer technique, mice were fed for one week chow enriched with $[\text{}^3\text{H}]$ cholesterol and had retinal cholesterol uptake to be roughly 3.6 % of total cholesterol concentration [141].

Simvastatin, a semi-synthetic derivative of lovastatin and HMG-CoA reductase inhibitor, was reported to have better bioavailability compared to other statins (within six weeks) in the murine neural retina and levels were considerably higher there than in the

brain. Adult mice given this therapy showed a considerable drop (approximately 20% after six weeks) in retinal cholesterol, as well as a reduction in sterol intermediates; otherwise, total retinal cholesterol uptake was unaffected. Systemic simvastatin administration resulted in partial suppression of retinal HMGCR activity [183, 196], based on the predicted turnover rate of cholesterol (about 54 days) and the proportion of endogenous (retina-derived) production compared to the overall retinal cholesterol reservoir (about 72%), these results confirmed specific activation of the retinal mevalonate cascade.

A different *in vitro* study with [³H]water showed *de novo* retinal cholesterol and dolichol synthesis in the frog. Radioactive [³H]acetate-labelling and detection using Radio-HPLC (High-performance liquid chromatography) identified *de novo* precursors [197]. The results indicated that most of the [³H]acetates were converted to squalene, not to sterols, and that the frog retina has a considerable steady-state level of squalene mass, unlike other vertebrates. As a result, the acetate is trapped in the squalene pool and does not participate in the formation of new cholesterol molecules. Cholesterol synthesis was measured at 3.4 pmol/h, indicating that retinal *de novo* sterol biosynthesis in amphibians (poikilotherms) is lower than in homeotherms (warm-blooded animal species). *In vivo* studies of frogs using intravitreal injection of [³H]acetates confirmed these results. Uptake of acetyl CoA to the mevalonate pathway is restricted due to its hydrolysis [198]; nevertheless, [³H]acetate was shown to be incorporated mainly into [³H]squalene and [³H]cholesterol, which were found both in whole retinas and isolated rod OS [199]. These findings relate to total retinal sterol production, but they do not examine or consider that a proportion of the sterols used by retinal neurons, notably photoreceptor cells, may potentially come from other retinal cells, such as Müller glia cells [200, 201]. Furthermore, regarding the mevalonate pathway in retinal cells, cell-specific studies investigating its role in retinal sterol homeostasis may provide crucial insights into the pathways.

The next discussion relates to the activity of the post-squalene branch of the mevalonate cascade in the neural retina. Pharmacological targeting of the Kandutsch-Russell or Bloch pathways can be carried out in order to evaluate the effect on retinal structure and function. Phenotypes are similar to the ones of rare genetic disorders characterized by low cholesterol production and an accumulation of precursors [157]. SLOS, the most frequent recessive genetic disorder affecting the mevalonate pathway, is a good example of this. Accumulation of 7DHC in biological tissues and fluids is the primary biomarker of this

condition, which is clinically defined by dysmorphologies such as 2–3 toe syndactyly, craniofacial abnormalities, cognitive deficits (autism spectrum), and rod and cone functional impairment [202-205]. The photoreceptor defects prove that cholesterol is essential for appropriate retinal function. The retina of the SLOS rat model shows significant accumulation of 7DHC, with a mole ratio of around 5.0 (7DHC/Cholesterol), compared to < 0.1 in controls. Elevation of the steady-state 7DHC level was linked to progressive, photoreceptor-specific retinal degeneration, activating a cell death mechanism independent of the caspase-3 pathway. Features of retinal degeneration that were observed included the presence of pyknotic nuclei, gradual rod OS shortening, shrinking of the photoreceptor layer, impaired clearance of shed rod OS by the RPE, and minimized and delayed response to light, suggesting both rod and cone dysfunction [206-208]. 7DHC is an intermediate molecule in the mevalonate pathway that shows extreme susceptibility to oxidation [209]; thus, production of a variety of toxic oxysterol metabolites may contribute to this disease [210-212]. The SLOS model can be pharmacologically and systemically treated with an inhibitor of DHCR7, known as AY9944 [trans-1,4- bis(2-dichlorobenzylamino-ethyl) cyclohexane dihydrochloride].

Targeting of DHCR7 with drugs has also been performed to test the effect on retinal cholesterol uptake. Sprague-Dawley rats treated with AY9944 were fed a cholesterol-free diet until postnatal day 28; rats were then separated into two groups, one of which was maintained on a cholesterol-free diet, while the other received chow-based nutrition containing 2% cholesterol, until postnatal day 74 [213]. For rats fed on the cholesterol-free diet, the major sterol expected component was 7DHC; however, cholesterol should be the predominant sterol in the retina of rats maintained on the cholesterol-rich diet. Conversely, under these conditions, the overall sterol level in the retina did not alter much, indicating that 7DHC was replaced at a 1:1 molar ratio in the retina via diet-derived, blood-borne cholesterol. This was a surprising result since it had been expected that the blood-retina barrier would prevent cholesterol uptake from the blood by the retina, in a similar manner to the blood-brain barrier. The mole ratio of 7DHC/Chol was 7.30 for the cholesterol-free group and 0.1 for the cholesterol-rich group [213]. These results were consistent with results from the [^2H]cholesterol-treated metabolic group described above. Altogether, these results demonstrated that blood-borne cholesterol can pass through the blood-retina barrier and can also be absorbed by the neural retina [141, 213]. Another method, utilizing intravenous injection of human LDL particles "doped" with cholestatrienol [cholesta-5,7,9(11)-trien-3-

ol], a biologically fluorescent derivative of cholesterol, was used to confirm these results; postinjection uptake of cholestatrienol into the retina was analyzed by confocal fluorescence microscopy. Rats were also injected intravenously with human LDL molecules containing [²H]cholesterol, uptake of which was monitored using LC-MS (Liquid chromatography–mass spectrometry) analysis of extracts of retinal lipid. Two hours after injecting derivatives of LDL, the choroid, RPE, and distal outer neural retina demonstrated cholestatrienol fluorescence; fluorescence was then detected in the outer retina, including photoreceptor cells by four hours, and throughout the entire neural retina after 6 hours. LC-MS revealed that the level of [²H]cholesterol in the neural retina increased with time [139]. Taken together, these different lines of data clearly demonstrated that cholesterol contained within LDL particles in the blood may be absorbed by the retina and dispersed widely throughout the neural retina.

In vivo, pharmacological and metabolic techniques have previously revealed insights on cholesterol biosynthesis and uptake in the adult neural retina, particularly in rodents. The retina is mainly composed of neuronal and glial cells, which are organized in distinct histopathological layers; however, sterol homeostasis in these layers has not yet been fully addressed. Hallmark proteins involved in sterol biosynthesis and the lipoprotein uptake pathway are expressed in the neural retina. Quantitative RT-PCR analysis revealed biosynthesis of pre-squalene and post-squalene enzymes, such as *MVK*, *HMGCR*, *DHCR24*, and *DHCR7* [145, 196] in this tissue. Furthermore, immunohistochemistry studies on the murine retina indicated higher levels in the INL than in the ONL of the retina. Ninety-day-old human retinal organoids derived from human embryonic stem cells have been used to undertake single-cell RNA-Seq studies [214], using the CRX (cone-rod homeobox gene) promoter to induce GFP expression. The *CRX* gene is expressed in developing precursors, as well as in mature photoreceptor cells and in bipolar cells [215-217]. *HMGCR*, *SQLE*, *MVK*, and other mevalonate pathway participants were significantly overrepresented in CRX-negative cells, whereas GFP-positive cells (photoreceptors) exhibited a low level of expression of mevalonate pathway genes [214], most likely due to the need for *de novo* synthesis of non-sterol isoprenoids, *e.g.*, dolichols [186]. In addition to these models, recent *in silico* modeling of retinal sterol homeostasis has revealed that photoreceptors obtain sterol externally rather than through the mevalonate pathway [218]. In conclusion, these findings show that *de novo* cholesterol production in photoreceptors is probably limited, despite the rapid pace of membrane building and turnover in photoreceptor OS membranes, which

necessitates a constant supply of lipids, including cholesterol, and proteins [134, 219]. It is also worth noting that a significant amount of lipid biosynthesis is required throughout retinal growth and maturation.

Future studies on sterol homeostasis during retinal development might lead to new understanding of congenital diseases related to sterol homeostasis. The use of specific genetic deletion techniques to examine retinal neuronal and glial-specific regulation of the mevalonate pathway should also provide insight into neuron-glial relationships in the retina, which contribute to the maintenance of balanced levels of cholesterol in the retina.

1.8.1.1. Mevalonate pathway activity in RPE Cells

Despite the demonstration of the presence of HMGCR in human and murine RPE cells [145, 196], analysis of RPE cholesterol production rates *in vivo* is complex due to the technical challenges associated with metabolic methods and the requirement for targeting suppression of the mevalonate pathway in specific cell types. Thus, RPE *in vitro* models of hereditary diseases involving the mevalonate pathway, associated pharmacological models, and traditional metabolic methods were used as alternative ways of examining *de novo* sterol biosynthesis in RPE cells. An *in vitro* model of SLOS had been produced using RPE derived from human induced pluripotent stem cells (iPSC). The characteristic feature of this model was reduced 7DHC enzyme level affecting cholesterol synthesis. SLOS-RPE cells derived from patient iPSCs were compared to iPSC-derived RPE cells from healthy human controls and cells were grown in delipidated serum. Compared to controls, SLOS-RPE cells showed higher steady-state levels of 7DHC (40 % of total sterol content). This study showed that the cholesterol synthesis pathway was activated [208]. *In vitro* incorporation of radiolabeled acetate into cholesterol in ARPE-19 cells, an immortalized human RPE-derived treated cell line, supported these data [142]. *In vivo* research using RNA-Seq suggested that the activation of mevalonate pathway genes including *Hmgcr*, *Dhcr24*, and *Sqle* fluctuates during the day in the RPE of 10- to 13-week-old mice [220]. These findings show that RPE cells can manufacture cholesterol on their own, but do not allow for the analysis of absolute sterol synthesis rates.

A summary of cholesterol the pathway in the retina is shown in figure 8.

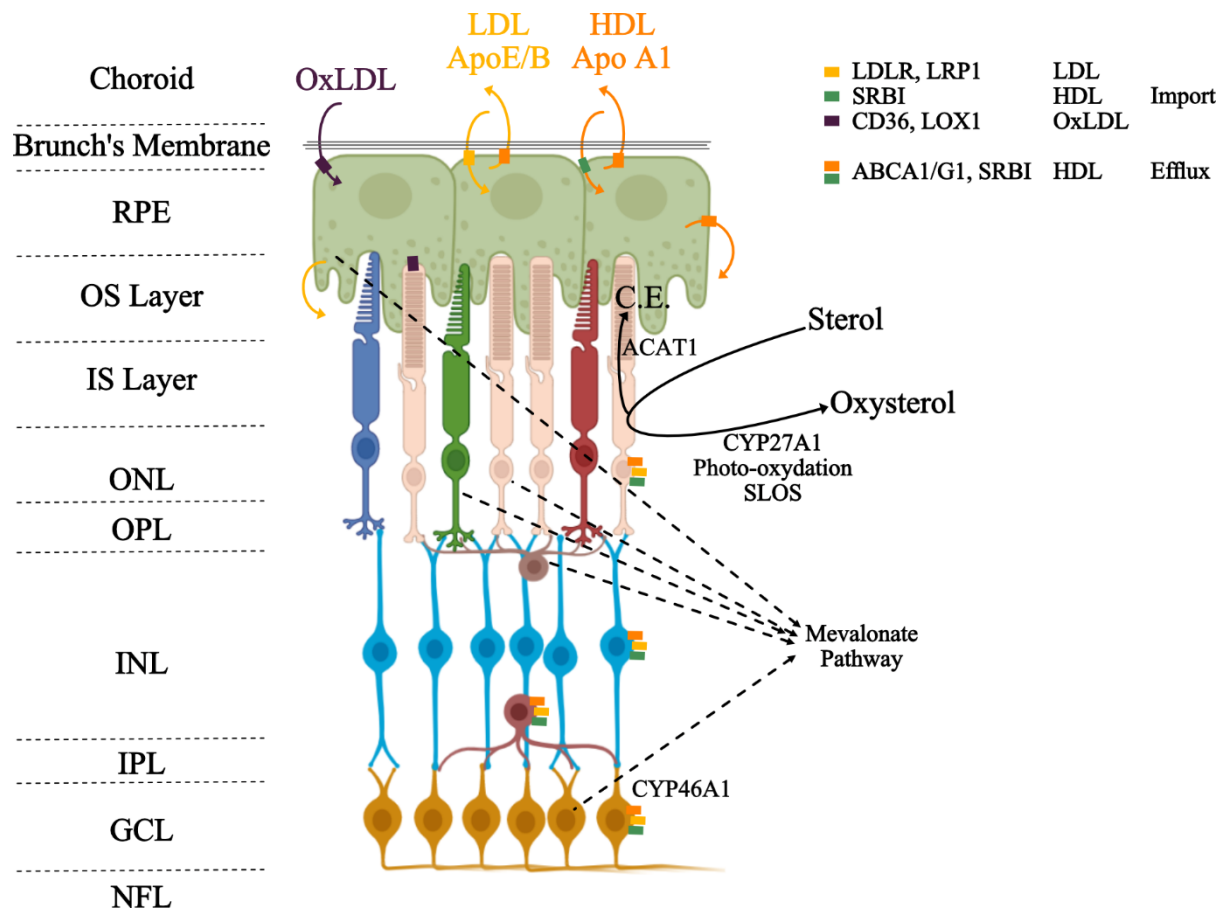


Figure 8. Model of cholesterol homeostasis mechanisms in the vertebrate retina. The cells of the retina synthesize but also uptake cholesterol. The mevalonate pathway is active in both the RPE and the neural retina. Cholesterol is actively synthesized, and excess of retinal cholesterol may be esterified and stored as lipid droplets by the activity of ACAT or package with APO proteins and secreted through ABCA1. Secreted cholesterol can then be uptake by neighbouring neurons in a LDLR-dependent way. The RPE exhibits a CD36-dependent uptake of OxLDL from the choroid. Moreover, CD36 is involved in diurnal uptake of rod OS tips. Modified and adapted from [138].

1.8.2. Uptake of Cholesterol in the Retina

RPE cells have an active role in retinal cholesterol circulation (Figure 9). Cholesterol can be synthesized in these cells and cholesterol efflux can occur as well. RPE cells also perform specialized cholesterol uptake. Cholesterol uptake from blood-borne LDL particles by RPE cells was first observed using Rhodamine-labeled LDL [221]. *In vitro* studies on ARPE-19 cells demonstrated LDL uptake via LDLR [139, 221]. Additionally, RPE-mediated uptake of LDL was shown using intravenous injection of human LDL in rats by confocal microscopy analysis of the retina with a labelling of human APOB [139, 222]. However, serum depletion in ARPE-19 cell can lead to transcriptional activation of mevalonate pathway genes [223]. In human and monkey retinas, expression of LDLR was observed in RPE cells, ganglion cells, and retinal vasculature endothelial cells [139, 145,

196]. Filipin, an inartificial fluorescent macrolide antibiotic compound that attaches to sterols, was used to identify cholesterol in different cell types in *Ldlr*^{-/-} knock-out mice. These mice showed abnormal retinal response based on electroretinogram (ERG) and an accumulation of esterified cholesterol on the basolateral surface of RPE cells, indicating poor LDL uptake and confirming that it is mediated by LDLR [224].

The analysis of the retinal phenotypes in patients allowed the evaluation of cholesterol in RPE cells. Mutations of NPC-I genes block free cholesterol transfer from lysosomes to the ER, causing accumulation of free cholesterol in the RPE and OPL of the retina [225, 226]. Additionally, a genetically modified NPC-I mouse model was characterized by a decrease of dark adaptation depending on age, as well as progressive specific loss of photoreceptor cells [225, 227]. Using spectral-domain optical coherence tomography in NPC-I patients, retinal neurons showed severe nerve fiber layer erosion and axonal degeneration [228]. Lysosomal acid lipase (LAL) is an essential enzyme for cholesterol homeostasis in the RPE cells [229, 230]. Remarkably, LAL deficiency causes the accumulation of lysosomal cholesteryl esters, but patients with this deficiency do not develop retinopathies.

The scavenger receptor class B type 1 (SRB-I) and the scavenger receptor class B type II (SRB-II) are multiligand membrane receptor proteins involved in cholesterol influx and effluxes, such as uptake of the HDL and OxLDL. Binding studies in bovine retina have shown that cholesterol uptake can occur via LDLR, SRB-I, and SRB-II in RPE cells [231]. Primary human RPE cells also express SRBs [232]. In monkey retinal neurons, differentially expressed APOs and SRBs were discovered in a comprehensive study and, although the presence of APOA-1, SRB-I, and SRB-II showed immunoreactivity in both the RPE and the GCL, photoreceptors exclusively expressed SRBs [139]. These results suggest complex intraretinal lipoprotein transport pathways regulating the maintenance of sterol homeostasis in the neuronal retina and RPE cells [139, 232].

SRB-I is crucial in the uptake of cholesteryl esters from HDL particles by cells and also the uptake of lutein by the RPE [233]. SRB-I is also involved in the efflux of cholesterol from extrahepatic tissues, hence it has both uptake and efflux functions [234]. For a better understanding, an SRB-I knock-out animal model was used to characterize SRB-I retinal function. When compared to controls, SRB-I mutant mice on a regular diet showed moderate reductions in dark-adapted a-wave and b-wave ERG responses. However, they did not

demonstrate visible retinal structural abnormalities [235]. Disorganization of the ONL, scarce sub-RPE lipid accumulation, ERG abnormalities, and considerable thickness of Bruch's membrane were all observed in mice fed with high-fat food. Hypolipoproteinemia and high blood cholesterol levels were observed in this animal model with a diet rich in high-fat nutrients.

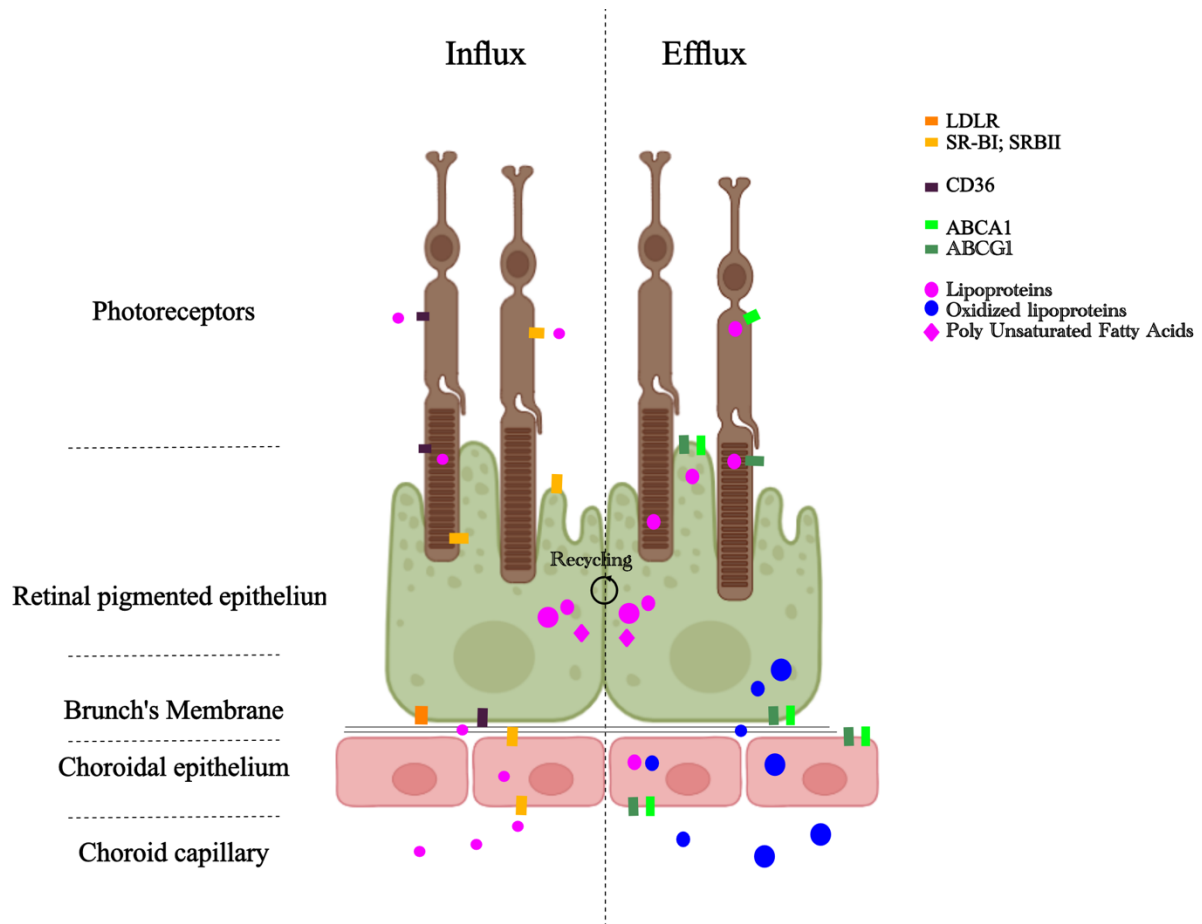


Figure 9. Cholesterol trafficking in the retina. The retinal pigment epithelium mediates transfers of lipids and lipoprotein particles (pink) between the circulation and photoreceptors. Scavenger receptors, SR-BI and SR-BII, cluster of differentiation 36 (CD36) and LDL receptors (LDLR) are involved in RPE lipids import and are shown on the left-hand side in yellow, orange and violet respectively. ATP binding cassette subfamily A member 1 (ABCA1) and ATP-binding cassette sub-family G member 1 (ABCG1) are involved in RPE lipids efflux and are shown on the right-hand side in green. Moreover, the RPE cells continuously recycle the lipids that are phagocytosed from photoreceptors outer segment membrane discs. The oxidized lipid species are either exported into the circulation as newly assembled lipoprotein particles (blue) or deposited into the basal extracellular space. Modified and adapted from [236].

1.8.3. Efflux of Cholesterol in the Retina

Much like cholesterol uptake, the cholesterol efflux mechanism is significant in the retina. Efflux mechanism works as collector of cholesterol by-products and is needed for retinal cholesterol homeostasis. RPE cells and neural retina cells express ABCA1 and ABCG1 [145, 237, 238] and all known mechanisms and components for cholesterol efflux

(LXR, ABC transporters, APOA-1, APOE, APOB). Participants in intracellular sterol trafficking (NPC-I, translocator protein (TSPO)) are also expressed in human and mouse RPE/choroid [144]. In this section, ABC transporters will be discussed as the leading players in retinal cholesterol efflux.

ABC transporters form a huge family of proteins and are mostly related to sterol transport, which is needed in all types of cells. ABCA1 and ABCG1 contribute to most cellular sterol efflux, depending on tissue expression levels [161]. ABCA1 and ABCG1 are widely expressed in most tissues, including the retina [169]. On the other hand, ABCG4 is mainly expressed in the brain [239]. In addition to efflux of sterols generated from OxLDL to HDL, macrophage ABCG1 have a protective function in atherosclerosis [240]. In one study, a considerable amount of cholesterol accumulation was observed in brain tissue from *Abcg1/Abcg4* double-knockout mice by eight months of age confirming the crucial role of ABCG1 and ABCG4 in cholesterol efflux in the brain [239]. Expression of ABCG1 and ABCG4 was found in all neural retina layers and RPE cells [169, 238, 239]. While no abnormality could be detected in the retina of *Abcg1/Abcg4* double-knockout mice, these animals showed mild retinal dystrophy with some accumulation of lathosterol [239].

In another study, the neural retina treated with an LXR- α agonist (T0901317) increased ABCA1 and ABCG1 expression, confirming the essential role of LXR- α and ABC transporters in regulating retinal sterol efflux [196]. Moreover, rod photoreceptor specific *Abca1* knockout (using a RhoiCre) mice exhibited the accumulation of lipid droplets [241]. Significant lipid accumulation could be detected in mice at one year of age and retinal age-related degeneration was observed. The *Abca1/Abcg4* knockout displayed higher amounts of retinal cholesterol, 7KChol, 24-, 25-, and 27-OH-Chol, when fed with a high-fat diet, lipid accumulation and retinal degeneration occurred faster than in the control group [242]. LXR- α loss of function causes similar outcomes, *i.e.* age-related retinal degeneration and cholesterol accumulation with an ABC transporter activity drop [243]. On the contrary, knockout of LXR- β did not result in photoreceptor dysfunction even 10 months after birth. Meanwhile, a deficiency of LXR- α or LXR- β may also cause lipid accumulation in RPE cells [243]. Additionally, the loss of LXR- β affected ganglion cell viability with reduced retinal aquaporin-4 production and microglial activity, both signs of neuroinflammation [244]. Surprisingly, suppression of cholesterol efflux caused a gradual retinal degenerative phenotype that appeared approximately at one year of age in mice. This is in contrast to

statin-induced suppression of *de novo* cholesterol biosynthesis or to the AY9944-induced model type of SLOS, which causes retinal degeneration over weeks [207]. In conclusion, these findings independently show effects on retinal degeneration even if with high variance in the rates of retinal sterol biosynthesis and turnover [141, 213].

The RPE cell layer plays a significant function in retinal cholesterol homeostasis. Dynamic cholesterol circulation was demonstrated in RPE cells. RPE and ARPE-19 cells both produce microsomal triglyceride transfer protein and APOB, indicating that they can build lipoprotein-like molecules on their own, most likely for transport purposes [245]. ARPE-19 cells grown in [³H]oleate produced [³H]-labeled cholesteryl esters and triglycerides in the culture medium, and the lipoprotein-like particles recovered from the culture media displayed physical properties equivalent to plasma lipoproteins [245]. The expression of APOA-1 has been found in the human and monkey RPE, especially in the neural retina [139, 246]. Double deletion of ABCA1 and ABCG1 in the RPE causes an accumulation of cholesteryl ester-rich lipid droplets in the RPE, as well as degeneration of the retinal neurons at six months. RPE-specific *Abca1* loss of function was sufficient for the accumulation of lipid droplets, indicating that ABCA1 plays a significant role in RPE cholesterol efflux [237]. These data are sustained by the finding that probucol, a strong bisphenol antioxidant and ABCA1 blocker, could decrease cholesterol efflux from RPE cells [144]. These studies suggest that, upon both apical and basolateral ends of the cell, polarized RPE can participate in ABCA1-mediated sterol transfer to APOA-1. Moreover, in an APOA-1-dependent way, RPE cells may efflux cholesterol generated from photoreceptor OS membranes on both the apical and basolateral sides of polarized RPE. Unfortunately, weakly polarized ARPE-19 cells are a poor *in vitro* model because failed to promote basolateral cholesterol efflux to APOA-1. These results imply that RPE cells have a substantial role for effective cholesterol efflux through the blood-retinal barrier [144]. Cholesterol efflux system mediated by ABCA1 and ABCG1 is presented in figure 10.

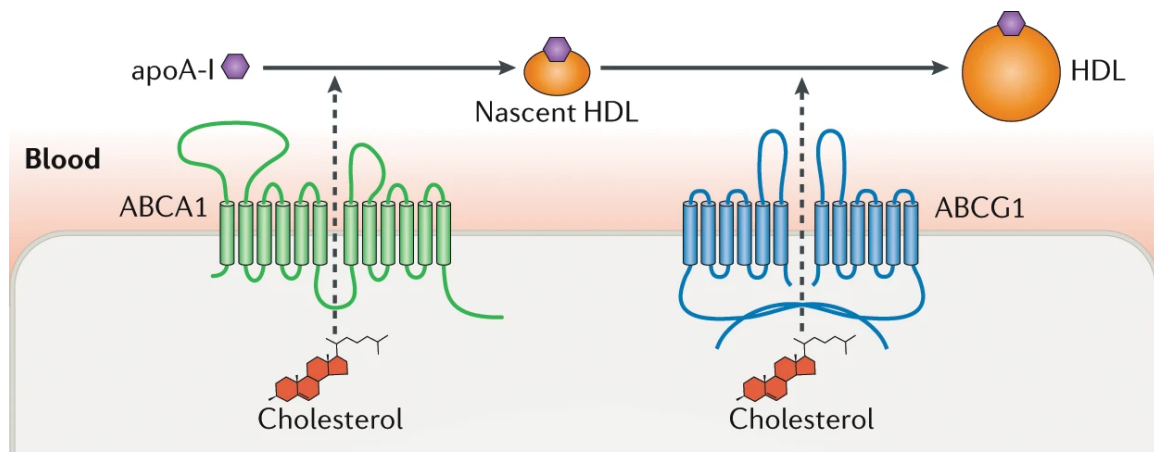


Figure 10. Cholesterol export is mediated by ATP-binding cassette (ABC) transporters. ABC subfamily A member 1 (ABCA1) and ABC subfamily G member 1 (ABCG1) are expressed on the cellular plasma membrane and transfer intracellular cholesterol to apolipoprotein A-I (apoA-I) in the blood. ABCA1 mediated transport of cholesterol to apoA-I generates nascent high-density lipoprotein (HDL). Subsequently, the nascent HDL accepts cholesterol exiting through ABCG1 and mature HDL is formed. Modified and adapted from [247].

1.9. The Role of Cholesterol in Photoreceptors

In most animal cells, cholesterol is a necessary membrane component and has been linked to many different cell processes which are critical for cell survival, such as enzymatic activity, cell division, cellular permeability, and receptor-mediated cellular processes. The mechanism of cholesterol homeostasis in the plasma membrane has been investigated extensively for decades because cholesterol has a significant function in the membrane. Today, the diverse impacts of cholesterol on the cell membrane are well established. Cholesterol is vital to maintain the basic features of the phospholipid bilayer and interacts with specific membrane proteins [248]. Cholesterol-protein binding is involved in managing membrane cholesterol interaction [249]. The properties of cholesterol have been studied in various phospholipid bilayer models establishing that cholesterol changes the characteristics of the bulk bilayer phase when interacting with the phospholipids and improves bilayer thickness [250] and lowers the permeability of the membrane [251].

Cholesterol affects the Na^+ gradient maintained by the plasma membrane. The cellular electrochemical gradient is generated through the Na^+/K^+ ATPases pump mechanism, and the plasma membrane maintains this critical gradient with a consume of ATP. When the membrane contains cholesterol, the leakage of Na^+ through a bilayer containing both phospholipids and proteins can be partially restrained [252]. It has been postulated that the role of cholesterol in the plasma membrane is to limit the depletion of the Na^+ gradient to save metabolic energy [9].

Yeagle described how cholesterol expands the gel to liquid crystal phospholipid bilayers' phase transition temperature [253]. Cholesterol regulates membrane fluidity by modulating hydrocarbon packing. This finding contributed to the idea that cholesterol influences the shift from lipid gel to the liquid crystalline phase in plasma membranes. Cholesterol has been hypothesized to play the role of the primary regulator of the "fluidity" of the bilayer hydrocarbon area in the membrane. Although "fluidity" is a vague phrase for describing membrane movements, "fluidity" in this context often refers to motional flexibility and hydrocarbon side chain packing. Cholesterol alters the dynamics of the bilayer hydrocarbon region [136].

Cholesterol can regulate membrane protein activity (such as Rhodopsin) via its effect on lipid packing [248]. Mammalian cells are often not healthy when utterly devoid of cholesterol as the structural properties of cholesterol discussed above are required for a functional plasma membrane. Other sterols may mimic some of the cholesterol membrane properties and replace cholesterol within this specific function. If the cell membrane cholesterol is reduced but not abolished, another sterol can replace the bulk membrane cholesterol and restore membrane function [254].

Focusing on the role of cholesterol in photoreceptors, cholesterol is one of the main components of the photoreceptor OS. Rod photoreceptor cells provide a unique model for studying cholesterol production, transportation, and activity in a single differentiated cell. Several methods have been used to explore the production, cellular distribution, and influence of membrane cholesterol on key functional parameters in this cell type. Rods can be used for studies in biophysics, particularly on the effects of cholesterol on rhodopsin, a model for the G-protein coupled receptor (GPCR) family [249]. Cholesterol, in fact, is linked with GPCR regulation [255]. This includes the GPCRs for nicotinic acetylcholine [256, 257], oxytocin [254, 258-260], cholecystokinin [259], serotonin [261], and transferrin [262]. Consequently, investigating how cholesterol affects rhodopsin function is expected to shed light on various GPCRs. In summary, cholesterol is a significant plasma membrane component that exists in abundant amounts in rod photoreceptors. This section examines the relationship between cholesterol and the rod photoreceptor OS in plasma and disk membranes.

1.9.1. The Rod Outer Segment Membranes and Cholesterol

The rod OS has a simple structure; however, functional features are more complex than the structure. The two membranes of the rod OS are plasma and disk membranes. The disks are flattened membrane vesicles that have been packed along the length of the OS and are enclosed by the plasma membrane. Despite its apparent simplicity, the rod OS is highly structured, with a complicated arrangement of lipids and proteins. The plasma and disk membranes have different functions that are represented in their lipid and protein contents (See Section 1.3). The disk membrane is where light is absorbed, and visual transduction begins. The plasma membrane, on the other side, maintains baseline ion permeability in the dark and hyperpolarizes in response to the phototransduction activation.

1.9.1.1. Plasma membrane Features

The lipid and protein content of the plasma membrane has proved challenging to study as the plasma membrane contributes to less than 10% of the rod OS [263]. Rhodopsin, a GPCR, is the most abundant protein in the plasma membrane, accounting for approximately 40% of the total protein content [264, 265]. Additionally, rhodopsin is the only membrane protein found in both the plasma and disk membranes [266]. Increased cholesterol causes reduction of rhodopsin content at the plasma membrane affecting visual transduction initiation, this topic is discussed in section 1.9.3. The role of plasma membrane rhodopsin is still unclear, raising the possibility of other functions for this GPCR. Rod OS membrane potential is maintained by two critical proteins, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger [267] and the cGMP-gated channel [268]. The plasma membrane also contains the GLUT-1 glucose transporter [269].

1.9.1.2. Disk membranes Features

Disk membranes have been postulated to derive from the plasma membrane at the base of the OS [206, 270], even though this hypothesis has been questioned. Cryo-EM (Cryo-Electron Microscopy) of the outer layer membranes helped to generate a model for disk morphogenesis where basal disks are enclosed by the plasma membrane [271]. It is widely accepted that new disks are generated at the base of the rod OS and shifted toward the apical tip over approximately ten days in vertebrate retinas [272]. Older disks are released and surrounded with the RPE to be phagocytosed at the apical end of the OS. This process

constantly replenishes the rod OS with new disks [134, 273, 274]. As a result of this process, apical disks are a few days older than basal disks.

Scientists originally assumed that the lipid and protein contents of the disk and plasma membranes were identical because disks derived from the plasma membrane. Nevertheless, the protein and lipid contents of the two membranes differ. One of the most prevalent membrane proteins in the disk membrane is rhodopsin, and it accounts for approximately 90% of the membrane protein, which is significantly higher than the amount in the plasma membrane [275]. Rhodopsin is found in the disk central planar area [276]. Embedded rhodopsin exists in freshly generated disk membranes which will transit along the OS length [277, 278]. During the renewal process of a disk from the base to the apical tip, the natural structure of rhodopsin must remain stable. The native conformation of rhodopsin has a limited lifespan because of kinetic stability, but long enough for the disk life [279, 280]. Mutations might affect the kinetic of the stability of rhodopsin. When a mutation causes a short half-life for the receptor, the protein could be denatured during apical displacement, resulting in the degeneration of rod photoreceptor cells.

A previous study analysed the rhodopsin structure in bovine disk membranes using atomic force microscopy (AFM). In the centre part of the disk, rhodopsin was observed as relatively dense and heterogeneously dispersed, comparable to monomeric rhodopsin. A protein-free lipid ring surrounds rhodopsin in the centre area, separating it from the disk rim [276].

Additional proteins, ROM-1, and the ABC transporters such as ABCA4, are found on the disk rim and are critical for structural stability and OS replenishment. For the stability of the length of the rod OS, as previously mentioned, the RPE must phagocytose the older apical disks while novel disks replace at the OS base. The integral membrane proteins of disks and the disk lipid composition (rhodopsin and caveolin) do not change during apical displacement, but the phospholipid head-group composition is modified during this displacement [263, 281-283]

1.9.2. Cholesterol Distribution in The Outer Segment of The Rod Photoreceptor

Cholesterol is the most abundant sterol in the retina and shows a slow transformation in the rod OS. The cholesterol used in membrane synthesis is derived from the systemic circulation via the blood and cholesterol is recycled within the retina. A small amount of

cholesterol can also be synthesized in the retina. Cholesterol recycling may be similar to that of docosahexaenoic acid (DHA) a significant retinal fatty acid [284-286].

According to early studies on cholesterol distribution in photoreceptors, cholesterol is primarily localized in the plasma membrane and newly generated disks [287, 288]. Isolation of the plasma membrane showed approximately 30 mole percent cholesterol. This amount is similar to other plasma membranes [263]. The general cholesterol concentration of the rod OS was around 10 mol percent, and the disk membranes account for around 90% of the total OS membranes. However, cholesterol was found not distributed equally in the rod OS, because heterogeneous distribution was observed. The heterogeneous distribution of cholesterol in the rod OS was characterized when the disk membranes were separated from each other and when in bovine and rat retinas each disk membrane was found with a different amount of cholesterol [287, 288]. These studies indicated that cholesterol concentration in rod OS varies and is higher in the basal disks than in the apical disks [289].

In conclusion, the results presented above suggested a complicated, heterogeneous cholesterol distribution in rod OS. The level of cholesterol is high in the plasma membrane of the OS from base to apical tip. Newly generated disks at the OS base are rich in cholesterol. On the other hand, the cholesterol rapidly decreases when the disks are apically shifted. Changes in the headgroup of the phospholipid and acyl chain composition support the depletion of cholesterol in the disks during apical displacement.

1.9.3. Cholesterol and Rhodopsin Function

Membrane cholesterol content has a significant impact on protein activities at the membrane. At the base of the OS, immature disk membranes are generated. The new disks must detach from the plasma membrane to enable visual transduction but, at the apical tip of the OS, disks fuse with the plasma membrane to form packets that are phagocytosed by the RPE. The structure of the membrane must be suitable for the activity throughout this process to ensure functionality. The function of rhodopsin is regulated by disk membrane lipid composition and optimization of conformational changes required for visual signal transduction. Abnormal lipid composition in the membrane could affect visual signal transduction or possibly OS regeneration. The lipid composition of the disk membrane most likely enhances rhodopsin capacity to execute the structural changes essential for visual signal transduction. Several studies have been conducted on the effect of lipid composition

on rhodopsin. These experiments were carried out in regenerated rhodopsin–phospholipid bilayers, including suitable compositions and natural disk membranes [290-294].

1.10. Oxysterols Causing Age-Related Macular Degeneration

Age-related macular degeneration (AMD) results in central loss of vision due to abnormalities in the macula. AMD is a primary cause of vision loss in Caucasians [295]. Scientists estimate that this illness will double in frequency within the next 20 years [296]. AMD has multifaceted mechanisms. The most important potential risk factor for macular degeneration is ageing. The aetiology of macular degeneration is influenced by both hereditary and environmental factors. Once macular degeneration has progressed, it is divided into dry (geographic atrophy) and wet (choroidal neovascularisation, CNV) AMD. The wet form is a much less prevalent one (approximately 10%), however, it advances quickly and severely, causing 90% of blindness due to AMD. Although dry macular degeneration does not have a proper treatment, anti-vascular endothelial growth factor (anti-VEGF) treatment is beneficial for wet AMD. The underlying mechanism of this therapy is the prevention of choroidal neovascularization and protection of the visual system [295].

The excessive accumulation of RPE extracellular aggregates (drusen) and basal linear aggregates (BLinD) is a clinical characteristic of AMD [297]. Histopathological studies reveal that cholesterol is abundant in these aggregates. It's suspected that improper transportation of reverse cholesterol is involved in the development of this kind of macular degeneration [298-300]. In addition, RPE cells die in a progressive manner causing the loss of photoreceptors in the macula. We need to keep in mind that, as explained above, RPE cells maintain cholesterol homeostasis in the retina by delivering cholesterol to photoreceptor cells and discarding extra cholesterol into the choriocapillaris by reverse transport of cholesterol [142-144]. Furthermore, genes that are responsible for oxysterol production are primarily expressed in the RPE [145, 301, 302], where elevated levels of oxidative stress are activated [303]. Oxidation of cholesterol is thought to be induced by oxidative stress. Therefore, oxysterols can be actively generated in the RPE by reactions of enzymes and free radicals. Indeed, oxysterol compounds have been discovered and localized to the RPE. These findings suggest that 7-KC (7-ketocholesterol), 24S-HC (24S-hydroxycholesterol), and 27-HC (27-Hydroxycholesterol) may influence AMD.

A link between increased 7-KC and AMD is supported by the presence of elevated levels of 7-KC in old RPE and drusen. In addition, increased levels of 7-KC have been observed in the RPE in response to photodamage [304, 305]. Treatment with 7-KC of human [306] and porcine RPE cells [307] reduced cell survival, triggered cell death and enhanced generation of reactive oxygen species. One of the most prevalent oxysterols in oxidized low-density lipoprotein (ox-LDL) is 7-KC [308]. Inflammatory response is induced by ox-LDL in the RPE and choroidal endothelial cells [309]. Inflammatory cytokines such as interleukin-1 (IL-1), IL-6, IL-8 (CXCL8), IL-18, and tumour necrosis factor- α (TNF- α) [305-307, 310-312] are also produced by 7-KC treatment in RPE cells, via the pathways of MEK/ERK, Akt/PKB, PKC, and NF- κ B [305, 310, 312]. In human RPE cells, 7-KC also induced ER stress [313]. Intravitreal injection of 7-KC in rats caused disorganized and reduced apical microvilli of the RPE. It also caused apical microvilli separation from outer segments of the photoreceptor, resulting in abnormalities in phagocytosis of the OS [312]. Interestingly, defective or reduced RPE phagocytosis is an important contributor to AMD [314].

In healthy people, a local macrophage type called retinal microglia is found in the inner retina. Retinal microglia migrate to the outer retina when they are activated due to stress and ageing. In the outer retina, microglia cells exhibit proinflammatory and proangiogenic activities. The migration of microglia has been linked to a variety of retinal diseases, including AMD [315]. Experiments conducted in elderly mice demonstrate the colocalization of 7-KC with subretinal microglia. 7-KC is a chemoattractant that causes the migration of microglia to the subretinal area. This migration causes microglial activation with enhanced pro-inflammatory cytokines (IL-1, IL-6, IL-18, and TNF- α) and proangiogenic factors such as VEGF, PDGF-B (platelet-derived growth factor-b) and ICAM-1 (intercellular adhesion molecule 1). In support of these data, transplantation of 7-KC treated microglia could lead to choroidal neovascularization and endothelial migration was increased by conditional culture medium containing microglia treated with 7-KC [316].

While 7-KC appears to have a harmful role in AMD, 24S-HC serves a protective function. Björkhem (2006) found that 24S-HC could pass the blood-brain barrier to be delivered to the liver via the circulation of lipoproteins [317]. 24S-HC also has a role in the activation of nuclear transcription factors, including LXR- α and β , previously presented as transcriptional activators of *Abca1* in glial cells and neurons [318]. Another protein

modulated by 24S-HC is apolipoprotein E in astrocytes, which rises efflux of cholesterol and maintains cholesterol homeostasis [319]. Furthermore, 24S-HC can inhibit the formation of amyloid B in Alzheimer's disease models by blocking amyloid precursor protein trafficking [320]. In patients that suffer from AMD amyloid B formation has been found in the drusen [321, 322]. The mouse model of the disease demonstrated that an anti-amyloid treatment reduced the damage of the retina and delays loss of vision [323]. Even though the amount of 24S-HC in the plasma of AMD patients is higher than the controls [324], it may change in the retina and RPE of patients and be a compensatory response. As a result, scientists believe that 24S-HC potentially protects against AMD by increasing the transportation of cholesterol, as well as decreasing amyloid B production in the neural retina and RPE.

The oxysterol 27-HC is quickly oxidized to 27-COOH via CYP27A1, so it is unnoticeable in the neural retina and RPE [325], and no 27-HC difference could be detected in the plasma between patients and controls [324]. Nevertheless, there could be a link between 27-HC and the disease [142, 309]. One of the mitochondrial membrane proteins is the translocator protein (TSPO). TSPO transports cholesterol between the outer and inner mitochondrial membrane. Cholesterol metabolism begins in the inner membrane of the mitochondria via CYP27A1 enzymatic activity. In response, 27-HC stimulates LXR signalling, upregulating ABCA1, ABCG1/4, and apolipoprotein E expression, also promoting transport of cholesterol in the RPE cells [326]. TSPO is also expressed in human RPE cells and monkey choroidal endothelial cells. Its production was found exceptionally high in the murine RPE, but it was observed to drop during ageing. In RPE cells, knock-out of TSPO resulted in impaired efflux and accumulation of cholesterol. Agonists of TSPO (FGIN-1-27, emapunil (XBD173), and etifoxine) enhanced the efflux of cholesterol and elevated oxysterol metabolism. They also reduced oxidative stress and inflammatory responses caused by oxidized LDL [142, 309]. All these findings show that transportation of cholesterol mediated by TSPO and 27-HC potentially have a role in AMD pathogenesis.

1.11. Models for Research Applications

In order to reproduce the degenerative processes of photoreceptor degeneration, the use of suitable model systems is unavoidable. It is well known that most gene mutations causing RD in humans will also cause RD in mice, as the physiological processes of the perception of vision and light are evolutionarily conserved among a wide range of organisms [327]. Other animal models such as *Drosophila*, zebrafish, frogs and other mammals,

demonstrated to be useful for the study of mechanisms related to RD [328-330]. References related to models in mice and rats can be found in [331, 332]; for larger mammals such as cats, dogs and pigs are available in [333-335].

There are several mouse models for RD but the most commonly studied one is the “retinal degeneration-1”, *rd1* [336] bearing a loss of function mutation in the *Pde6b* gene, encoding for the beta subunit of PDE6 [337]. In this model absence of PDE6 protein [338], and to exceedingly high cGMP levels in rods were demonstrated [32, 61]. Excessive cGMP was linked to loss of the rods within the first two weeks after birth with a rapid retinal degeneration [339] and mutation-independent secondary death of cones [340]. In this murine model degeneration progresses extremely fast, thus, while it is very helpful for biochemical studies, it does not represent the progression of the disease in patients. A second model bearing a mutation in the *Pde6b* gene is the *rd10* mouse [341]. Loss of rods in the *rd10* retina is slower compared to the *rd1* model because the peak of rod cell loss is at about post-natal day 18 (PN18) in the *rd10* mutant retina while it is at around PN11 in the *rd1* mutant retina [11, 75]. In addition, more recent mouse models characterized by mutations in the *Pde6a* gene were generated [100, 342]. Of note is that those models carry point mutations (R562W, D670G, or V685M), leading to single amino acid changes in the PDE6A protein. Those mutations affect the PDE6A protein expressions and activity, and cGMP accumulation in rods is observed before the rod loss [100, 343]. Those animals allow to match patient genotypes and provide a model for the study of homozygous and compound heterozygous disease processes [100].

The primary rod degeneration can be linked to mutations in light-sensitive visual pigment rhodopsin. Rod degeneration is associated with abnormal expression, high levels, or absence of rhodopsin [344-346]. The P23H rhodopsin protein mutation is the most common form of blindness in patients of autosomal dominant RP [69]. P23H opsin protein accumulation was observed in the ER in transgenic P23H opsin animals [37, 67, 347] and caused ER stress [67, 348]. The knock-in *Rho*^{P23H/+} mouse model is the most commonly used model with the best mimicking genetic background as patients, showing similar pathological progression of the diseases like in patients [69].

In vitro models for research are commonly used by researchers. Several retina cell lines are available to study inherited eye diseases [349-352]. On the other hand, a limited number of photoreceptor cell lines exist; examples for the human cell lines are WERI-Rb1

and Y-79 [353-355]. The origin of these cell line is the retinoblastoma tumor. They have limited characteristics of rod photoreceptors while expressing some rod-specific genes [356]. Differentiation *in vitro* protocols of retinal stem cells into photoreceptors have been developed and demonstrated that these cells could be used for drug testing [84, 357, 358]. Nevertheless, this system is characterized by a high variability because primary cells need to be isolated from murine eyes, which is a considerable disadvantage.

661W is the cell line commonly used by researchers to study the retinal photoreceptors. The origin of the 661W cell line is a retinal tumor induced in transgenic mice, and it was derived from an immortalized photoreceptor progenitor. The transgenic mice expressed simian virus 40 (SV40) T antigen under the transcriptional control of the human interphotoreceptor retinol-binding protein (*IRBP*) promoter, a gene expressed specifically in rod and cone photoreceptors [359]. The 661W cell line is a suitable cell model for studying photoreceptor degeneration. It expresses photoreceptor precursor genes, such as cone and rod photoreceptor genes [360, 361]. 661W cell line has characteristics that make this cell line a promising model for RP. In fact, it can be grown as a monolayer, can be easily genetically modified *in vitro* and there is the possibility of inducing differentiation with specific treatments [66, 362].

2. AIM OF THE THESIS

This Ph.D. thesis aimed to identify gene expression changes during photoreceptor cells degeneration to find new biomarkers and possible targets for the development new therapeutic approaches. Currently, more than 100 different genes have been identified in RP patients, but even more genes are probably linked to RP beyond these numbers. The high genetic heterogeneity of RP hampers the development of treatments to cure RP. A more profound elucidation, at the molecular level, of the mechanisms underlying the progression of the disease is needed for the development of new treatments for patients. This study mainly focused on transcriptional changes during rod photoreceptor degeneration to gain insights into rod photoreceptor degeneration mechanisms.

The first aim was the development of a new *in vitro* model, based on the 661W cell line, able to mimic the *PDE6B* gene loss of function mutation in RP. We designed a procedure to induce the expression of rod-specific genes in 661W cells by genetic modification of the cell line with a transgene expressing the NRL transcription factor. After verifying that the 661W-A11 cell line acquired a rod-like phenotype, the cell line was treated with Zaprinas, a PDE6 inhibitor to induced cell death and activate pathways like the ones identified in RP mutant rod photoreceptors *in vivo*.

The second aim was to identify the differentially expressed genes during rod-like cell degeneration. The transcriptome analysis compared cell exposed to Zaprinas versus not treated cells. After identifying the differentially expressed genes in response to PDE6 inhibition, candidate genes were chosen for validation in animal models of RP. Interestingly, we found significant downregulation of genes mainly related to cholesterol biosynthesis and metabolism, a fundamental lipid for the membranes of the rod OS and, possibly, for rhodopsin functionality.

The final aim was to develop a differentiation protocol to generate *in vitro* mature rod-like cells that could provide a better model for drug screening. We were able to improve the cell model demonstrating increased expression of rod-specific markers and elongated morphology. We also achieved to mimic the disease in this improved model by treatment with zaprinast. Altogether, these data confirm that the newly generated cellular system will help the study of rod photoreceptor degeneration.

3. MATERIALS AND METHODS

3.1. List of Materials

Table 1. Reagents for Culture medium

Reagent	Company	Catalog number
Dulbecco's modified Eagle's medium (DMEM) 1g/L Glucose	Gibco	22320-030
Dulbecco's modified Eagle's medium (DMEM) 4g/L Glucose	Gibco	11960-085
Dulbecco's modified Eagle's medium (DMEM)/F-12	Gibco	3133-028
Fetal bovine serum (FBS)	Gibco	10270
Phosphate-buffered saline (PBS)	Sigma	D8537
Penicillin and streptomycin	Sigma	P0781
L-Glutamine	Sigma	P0781
Trypsin-EDTA	Gibco	25200056

Table 2. Reagents for Cell Differentiation

Reagent	Company	Catalog number	Stock Concentration	Final Concentration
Taurine	Sigma	T0625	50 mM	50µM
Sodium Butyrate	Sigma	B5887	400 mM	4mM
Basic fibroblast growth factor (bFGF)	Sigma	F3685	50µg/mL	10 ng/mL
Heparin	Sigma	H3149	27,32 mg/mL	2 ug/mL
Retinoic acid	Sigma	R2625	0.05 M	2uM
N-2 supplement	Gibco	17502048	100x	1x
Aphidicolin	Sigma	A0781	30mM	0,6 µM
CN03	Biolog	P007	20 mM	50 µM
Laminin from Engelbreth-Holm-Swarm murine sarcoma basement membrane	Sigma	L2020	1-2 mg/mL	1 µg/cm ²
Human recombinant laminin 521	Biolamina	LN521-02	1 mg/ml	3 ug/cm ²
Zaprinast	Sigma	Z0878	50mM	400 µM

Table 3. Chemical Reagent

Reagent	Company	Catalog number
Agarose	Sigma	A9539
Bovine Serum Albumin (BSA)	Sigma	A7906
Dimethyl sulfoxide (DMSO)	Sigma	D2650
dNTP Set (100 mM)	Invitrogen	10297117
Ethanol	Sigma	32205
Ethidium bromide	Sigma	E1510
Ethidium Homodimer-2 (EthD-2)	Invitrogen	E3599
Glycine	Sigma	G8898
Hydrochloridric acid	Sigma	320331
Isopropanol	Carlo Erba	4151560
Mowiol 4–88	Sigma	81381
MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide)	Invitrogen	M6494
Paraformaldehyde (PFA)	Sigma	158127
Platinum™ <i>Taq</i> DNA Polymerase	Invitrogen	10966018
Triton X-100	Sigma	T8787

3.2. Cell Culture Methods

661W cells are a cell line derived from a transgenic mouse line that was immortalized with the SV40 T antigen expressed under control of the *IRBP* promoter, specific for rod and cone photoreceptors (Tan et al., 2004). 661W cells were cultured in low glucose DMEM, supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 IU/ml penicillin and 100mg/ml streptomycin at 37°C in 5% CO₂ incubator.

For passaging, 1 ml of 0.25% trypsin-EDTA solution was added to 661W cells and a 1:10 dilution was done every 2-3 days. Cell aliquots were saved in liquid nitrogen in freezing medium (95% FBS, 5% DMSO).

3.3. Cell Differentiation

Sterile coverslips in 24 wells plates were coated for 2 hours with 300 μ l of 3 μ g/ml Laminin from Engelbreth-Holm-Swarm mouse sarcoma basement membrane (regular Laminin) or 5 μ g/ml Laminin 521 according to manufacturer's instructions. After one wash with PBS, 20000 cells were seeded in 300 μ l culture medium per well and incubated overnight. The following day, culture medium was removed, and differentiation medium was added. For differentiation, cells were incubated for 5 days in different formulations of differentiation medium:

- Differentiation medium 1 (DM1): DMEM low glucose supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml bFGF, 50 μ M taurine, 2 μ g/ml heparin, 4 mM sodium butyrate, 2 μ M retinoic acid.
- Differentiation medium 2 (DM2): DMEM low glucose supplemented with 10% FBS, 100 IU/mL penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml bFGF, 50 μ M taurine, 2 μ g/ml heparin, 4 mM sodium butyrate, 2 μ M retinoic acid and N2 supplement.
- Differentiation medium 3 (DM3): DMEM/F12 supplemented with 1% FBS, 100 IU/mL penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml bFGF, 50 μ M taurine, 2 μ g/ml heparin, 4 mM sodium butyrate, 2 μ M retinoic acid and N2 supplement.
- Differentiation medium 4 (DM4): DMEM/F12 supplemented with 100 IU/mL penicillin and 100 mg/ml streptomycin, 2 mM L-glutamine, 10 ng/ml bFGF, 50 μ M taurine, 2 μ g/ml heparin, 4 mM sodium butyrate, 2 μ M retinoic acid and N2 supplement.

3.4. Cell Viability Analysis (MTT Assay)

For cell viability analysis, 95 μ l of cell suspension containing 6,000 cells was plated in each well of a 96 well plate (Corning® Costar®) and cultured for 24 hours. The following day, different concentrations of the zaprinast, a PDE6 inhibitor, were dissolved in sterile DMSO. 5 μ l of every concentration or 5 μ l of DMSO, as control, were added to the culture medium in each well and incubated at 37°C in 5% CO₂ incubator for 24 and 48 hours. Cytotoxicity of zaprinast was assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric analysis. 10 μ l of MTT solution (5 mg/ml in PBS) was

added to each well and incubated for 4 hours at 37°C in 5% CO₂ incubator. The plate was centrifuged at 1800 rpm for 10 minutes and the supernatant was very carefully removed. 100 µl DMSO was added to each well to dissolve formazan crystals and shaken at 130 rpm for 15 minutes. Finally, absorbance was measured at 540 nm in a microplate reader (Labsystems Multiskan MCC/340, Fisher Scientific). Data derived from two independent experiments performed in triplicate wells.

3.5. Immunofluorescence

Culture medium was removed from cells cultured on coverslips (see chapter 3.3) and washed with 500 µl of PBS three times for 5 minutes. Cells were fixed for 15 minutes at room temperature (RT) with 2% PFA which was prepared in PBS. The cells were washed three times for 5 minutes with 500 µl PBS and permeabilized with 0.2% Triton-X100 in PBS for 10 minutes. After washing with PBS for five minutes, slides were incubated for 1 hour at RT with 300 µl of blocking solution (3% BSA in PBS). Next, blocking solution was removed and the cells were washed with PBS three times for 5 minutes. Incubation with 50 µl of primary antibodies diluted in PBS at the desired concentration (see Table 3) was performed overnight at 4°C. The following day, the cells were washed three times with 500 µl PBS for 5 minutes. Secondary antibodies were added together with 0.1 µg/ml DAPI (4',6-diamidino-2-phenylindole) both diluted in PBS (see Table 3) and the cells were incubated for 60 minutes at RT in the dark. After three washes with 500 µl of PBS for 5 minutes, coverslips were mounted with Mowiol 4-88 (Sigma) and analyzed at a Zeiss Axio Imager A2 fluorescence microscope.

Table 3. Antibodies

Primary Antibodies	Company	Catalog Number	Dilution
1D4 (Rhodopsin)	Santa Cruz	sc-7294	1:1000
Ac-alpha-Tubulin	Cell signalling	5335S	1:100
G3G4 (anti BrDU)	DSHB	AB_2314035	1:8000
GNAT1	Sigma	SAB-4501223	1:300
PDE6B	Abcam	ab5663	1:100
Peripherin	Millipore	ab1530	1:100

P-VASP (S239)	Nanotools	0047-100/VASP-16C2	1:200
Rhodopsin Kinase	Sigma	R3151	1:1000
Secondary antibodies			
AlexaFluor568 goat anti-mouse	Invitrogen	A11004	1:1000
AlexaFluor568 goat anti-rabbit	Invitrogen	A11011	1:1000
OregonGreen488 goat anti-rabbit	Life Technologies	6381	1:1000

3.6. Bromodeoxyuridine (BrdU) staining for cell proliferation analysis

After differentiation, cells were exposed for 3 hours to 10 μ M BrdU. After washing with 500 μ l of PBS, cells were fixed with 300 μ l of PFA 2% for 15 minutes at RT. The cells were washed with 500 μ l of PBS and incubate with 300 μ l of 2N HCl at 37°C for 30 minutes. The cells were, then, incubated with 300 μ l of 0.1 M Borate Buffer pH 8.5 for 15 minutes at RT and washed with 500 μ l of PBS. After blocking with 3% BSA, 1% glycine and 0.3% Triton-X100 in PBS for 30 minutes at RT, cells were incubated with anti-BrdU primary antibody in 500 μ l of PBS that contained 3% BSA and 1% glycine at 4°C overnight. Next, the cells were washed 3 times for 5 minutes with PBS and incubated with the secondary anti-mouse antibody and 0.1 μ g/ml DAPI for 60 minutes at RT. The cells were washed 3 times for 5 minutes with 500 μ l of PBS and the slides were mounted with Mowiol 4-88 and analyzed at a Zeiss Axio Imager A2 fluorescence microscope.

3.7. Ethidium homodimer (EthD2) cell death assay

Cells cultured on coverslips were washed in PBS and fixed with 2% PFA for 10 minutes at RT. Cells were washed with 500 μ l PBS three times for 5 minutes, stained for 2 minutes with EthD-2 diluted 1:1000 in PBS and washed again with 500 μ l PBS three times for 5 minutes. Incubation with 0.1 μ g/ml DAPI was performed for 60 minutes at RT in the dark. Then, the cells were washed three times with 500 μ l of PBS for 5 minutes and slides were mounted with Mowiol 4-88. The cells were analyzed using a Zeiss Axio Imager A2 fluorescence microscope. For each replicate, images of 10 different fields were acquired (139.51 μ m x 105.21 μ m), and dead cell quantification was performed by counting all EthD2-labeled cells in each field and dividing this value by the total number of cells, based on the nuclear DAPI staining.

3.8. Transcriptome Analysis

RNA was extracted from cell pellets that were collected after treatments or retinas of animal models using Qiagen RNeasy Plus micro Kit or Qiagen miRNeasy Mini Kit (for RT-PCR) according to manufacturer's instructions. Cell pellets or retinas were homogenized in 350 μ l of RLT plus Buffer or 700 μ l of QIAzol Lysis Reagent (miRNeasy Mini Kit) by vortex and pipetting.

The homogenized lysate in RLT plus Buffer was transferred to a gDNA Eliminator spin column placed in a 2 ml collection tube and centrifuged for 30 seconds at 8000 x g. One volume of 70% ethanol was mixed with the flow-through and transferred into a MinElute spin column placed into a 2 ml collection tube and centrifuged for 15 seconds at 8000 x g.

The homogenate in QIAzol Lysis Reagent was added with 140 μ l chloroform and shaken vigorously for 15 seconds and incubated 3 minutes at room temperature. After centrifugation, the aqueous phase containing RNA was transferred to a new collection tube and 1.5 volumes of 100% ethanol were added. The sample was loaded into a RNeasy Mini spin column in a 2 ml collection tube and centrifuge at 8000 x g for 15 seconds at room temperature.

The flow-through was discarded and 700 μ l Buffer RW1, 500 μ l buffer RPE and 500 μ l 80% ethanol were added to the column subsequently centrifuging after each addition for 15 seconds at 8000 x g. The column was finally placed into a new 2 ml tube and centrifuged 5 minutes at full speed with open lid to dry. RNA was eluted in 14 μ l RNase-free water after incubating the water 1 minute on the membrane and centrifuging 1 minute at full speed. RNA integrity was analyzed using the Agilent RNA 6000 Nano Kit (5067-1511, Agilent Technologies) and 4 replicates of each condition were sent to the company Next Generation Diagnostic srl (Pozzuoli, Italy) to perform RNA-Seq analysis.

cDNA was synthesized from 1 μ g of RNA using the HiScript III RT SuperMix for qPCR (+gDNA wiper) kit (R323 Vazyme Biotech, Nanjing, China). PCR was performed with 0.5 or 1 μ L cDNA using specific primers (see Table 4) under the following conditions: 30 seconds at 94 °C, 30 seconds at 60 °C and 30 seconds at 72 °C for 35 cycles. Amplified products were analyzed by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining with the BioRad ChemiDoc Imaging system. Real-time qPCR was carried out with the ssoAdvanced Universal SYBR Green Supermix (Bio-Rad) using 1 μ L of cDNA

diluted 1:2 in RNase free water using the CFX96 real-time PCR detection system (Bio-Rad) under the following conditions: 30 seconds at 95°C, and 39 cycles of 5 seconds at 95°C and 30 seconds at 60°C; the relative gene expression level was calculated using the ribosomal protein 26 gene *RPS26* as reference.

Table 4. Primers

Gene	Forward	Reverse
<i>RPS26</i>	AAGTTTGTTCATTCGGAACATT	GATCGATTCTTAACAACCTTG
GENERAL UPREGULATED GENES		
<i>Ypel3</i>	CAAAGACAACGGCTGGGACT	CCTTCAGGAGGAGCGAAACC
<i>Cdkn1a</i>	GCAAAGTGTGCCGTTGTCTC	CGTCTCCGTGACGAAGTCAA
<i>Hdac5</i>	TCCTCATCGTGGACTGGGAT	CCCCAGAGCCTGGAAAGAAG
GENERAL DOWNREGULATED GENES		
<i>Postn</i>	ACTCTGAGCATGGACCAACG	TCGGAGTTGTTTGCCTCCAA
<i>Il1rl1</i>	ATAAAAAGCCGCCAAGCTGC	ACCACTGAACAGGTGCTGTC
<i>Igf1</i>	GCTGGTGGATGCTCTTCAGT	TCCGGAAGCAACACTCATCC
<i>Nfatc1</i>	GCCTCGAACCCTATCGAGTG	AGTTATGGCCAGACAGCACC
<i>Hk2</i>	CATCACGCCAGTGACTCTGA	GGTCTAGCTGCTTAGCGTCC
<i>Axin2</i>	TGACTCTCCTTCCAGATCCCA	TGCCACACTAGGCTGACA
CHOLESTEROL UPREGULATED GENES		
<i>Acot2</i>	AAGTGCCTATGAAGGACTGAGG	TCTTACGGCACTGGGGAATG
<i>Abca1</i>	AGTAGCAGCACCGTGTCTTG	TGGTCAGCGTGTCACTTTCA
<i>Abca2</i>	AGCTGCTGCTCTGGAAGAACG	ATGAAGAAGAGGACAAGCGGG
CHOLESTEROL DOWNREGULATED GENES		
<i>Srebf2</i>	TGGGCGATGGATGAGAGCAG	TCTCCCACTTGATTGCTGACA
<i>Hmgcr</i>	TGATGGGAGCTTGCTGTGAG	TAGTGCTGGCCACAAGACAG
<i>Mvk</i>	TCGGCCTCACACAAACTCTC	TGATCTCCGAGAGAGGGCAA
<i>Nsdhl</i>	GTCCTCATGGCATTTTCGGC	GTCCACCAGGTTTTCCCAT
<i>Fdps</i>	GAGAAGGAACACGCCAATGC	TTGTCCTGGATGTCAGTGCC

3.9. Bioinformatics Analysis

Data were analysed by ROSALIND®, with a HyperScale architecture developed by ROSALIND, Inc. (San Diego, CA). Individual sample counts were normalized via Relative Log Expression (RLE) using DESeq2 R library [62]. Read Distribution percentages, identity heatmaps, and sample MDS plots were generated as part of the QC step. DESeq2 was also used to calculate fold changes and p-values and perform optional covariate correction.

Clustering of genes for the final heatmap of differentially expressed genes was done using the PAM (Partitioning Around Medoids) method using the fpc R library* Hennig, C. Cran-package fpc. <https://cran.r-project.org/web/packages/fpc/index.html> (. Hypergeometric distribution was used to analyse the enrichment of pathways, gene ontology, domain structure, and other ontologies. The topGO R library was used to determine local similarities and dependencies between GO terms in order to perform Elim pruning correction. Several database sources were referenced for enrichment analysis, including Interpro [363], NCBI [364, 365] , MSigDB[366] , REACTOME[367] , WikiPathways [368], BioPlanet, PANTHER, and BIOCYC.

. Enrichment was calculated relative to a set of background genes relevant for the experiment (<https://rosalind.onramp.bio/>).

3.10. Animal models

All procedures on mice were conducted at CSSI (Centro Servizi Stabulario Interdipartimentale) and approved by the Ethical Committee of University of Modena and Reggio Emilia (Prot. N. 106 22/11/2012) and by the Italian Ministero della Salute (346/2015-PR). For this project three animal models for Retinitis Pigmentosa were used for the validation of gene expression. C57/BL6J knock in mice with the P23H mutation in *Rhodopsin* gene *Rho*^{P23H/+} were obtained from the Palczewski lab [69]. Probing mechanisms of photoreceptor degeneration in a new mouse model of the common form of autosomal dominant retinitis pigmentosa due to P23H opsin mutations. J. Biol. Chem., 286, 10551–10567). C57/BL6J animals carrying *Pde6b* mutation (*rd10*) or C3H animals carrying *Pde6b* mutation (*rd1*) were kindly provided by Professor Paquet-Durand (Tübingen University). As control age-matched wild type (WT) C57/BL6J or C3H mice were used. Mice were maintained in a 12 h light/dark cycle and had free access to food and water. *Rho*^{P23H/+} were sacrificed at the age of 19 days after birth (postnatal day 19, PN19), *rd10* mutant mice were sacrificed at PN18, *rd1* mice were sacrificed at PN11 and the retinas were collected. The ages chosen for the analyses in each genotype were based on previous data reporting peak of cells death at PN19 for *Rho*^{P23H/+} [68]and at PN18 for *rd10* [11].

3.11. Statistical Analysis

For each experiment, data were obtained from at least 3 biological replicates and analyzed by unpaired Student's t-test using GraphPad Prism version 7 (GraphPad software,

San Diego, CA, USA). A value of $p \leq 0.05$ was considered significant. The average of each experiment was presented as means $\pm$ SD (standard deviation).

4. RESULTS

4.1. Generation of a New *In Vitro* System to Mimic Retina Degeneration

4.1.1. Generation and Selection of one 661W clone expressing NRL

With the aim of obtaining a stable expression of the rod specific NRL transcription factor, murine *Nrl* cDNA was cloned into the pLXSN vector, and the replication-incompetent retrovirus was used to transduce 661W cells. Transduced cells, that will be called from now on 661W+NRL, were selected according to neomycin resistance, and cells were analyzed by RT-PCR to determine their *Nrl* and Rhodopsin (*Rho*) gene expressions (Figure 11).

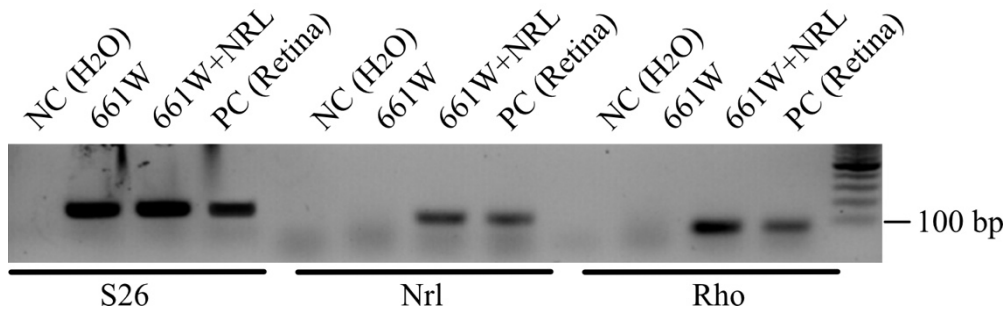


Figure 11. Analysis of expression of the rod-specific genes by RT-PCR in 661W and NRL modified 661W (661W+NRL). PC: positive control was cDNA from murine retina NC: negative control was a reaction without cDNA (retrotranscription -).

As shown in Figure 11, expression of *Nrl* and *Rho* genes was compared in 661W and 661W+NRL cells, which are the bulk of retrovirally transduced cells. The gene encoding the ribosomal protein RPS26 (*S26*) was also analyzed as a reference gene. Expression of *Nrl* and *Rho* was confirmed in 661W+NRL cells but not 661W cells.

In this model, single-cell clones were selected with a limiting dilution method. Five clones out of eleven were selected, depending on the primary expression of the Cyclic nucleotide-gated channel subunit alpha 1 (*Cngal*) gene (Figure 12). One clone, 661W-A11 showed the highest activation of *Cngal*.

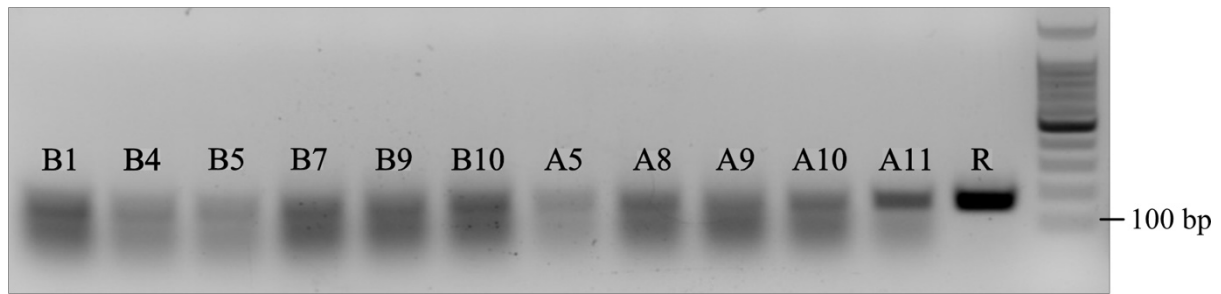


Figure 12. Analysis by RT-PCR of expression of the *Cngal* gene in 11 661W+NRL clones. *Cngal* is expected to be increased by overexpression of the NRL transcription factor. Five clones showed differential expression of the rod-specific *Cngal* gene when analyzed by RT-PCR, and RNA from retina was used as a positive control (R). Clone 661W-A11 gave the most convincing expression of *Cngal*.

However, not all clones could be maintained successfully *in vitro*. Therefore, depending on the expression of rod-specific genes and the *in vitro* expansion properties, clone A11 was selected and was called 661W-A11.

After that, the expression of other NRL target rod-specific genes was confirmed by real-time qPCR in clone 661W-A11. Such genes included *Rho*, G protein subunit alpha transducin 1 (*Gnat1*), cyclic nucleotide-gated channel subunit beta 1 (*Cngb1*), phosphodiesterase 6B (*Pde6b*), and nuclear receptor subfamily 2 group E member 3 (*Nr2e3*) (Figure 13).

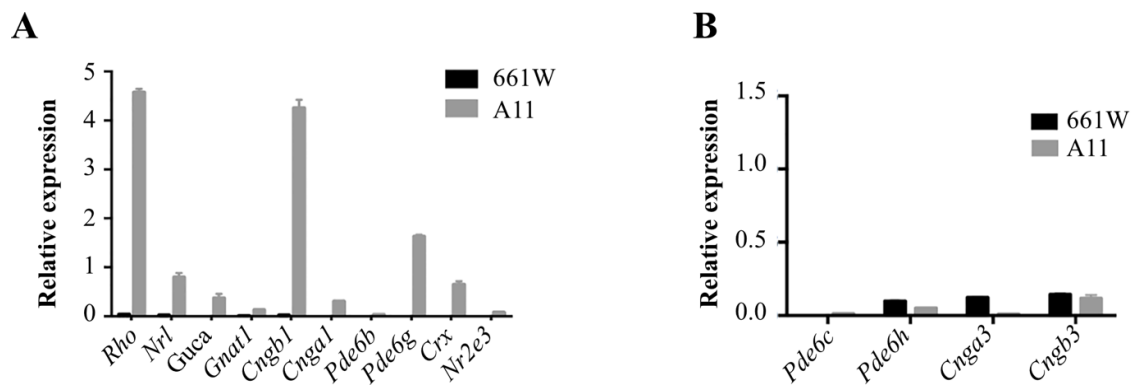


Figure 13. Expression of rod-specific (A) and cone-specific (B) genes were analyzed by real-time qPCR in 661W and 661W-A11. *RPS26* was used as a reference gene. 661W-A11 cells showed a significant upregulation of rod-specific gene expression compared to 661W cells but a similar expression of cone-specific genes.

4.1.2. Characterization of 661W-A11 Cells

The 661W-A11 clone was further characterized by immunofluorescence analysis, which confirmed higher protein expression for RHO, PDE6B and GNAT1 (Figure 14). When the morphology of 661W-A11 was compared with the one of 661W cells, 661W-A11 cells displayed a more elongated shape and a lower replication rate. These characteristics suggested that clone 661W-A11 gets features of the rod photoreceptor.

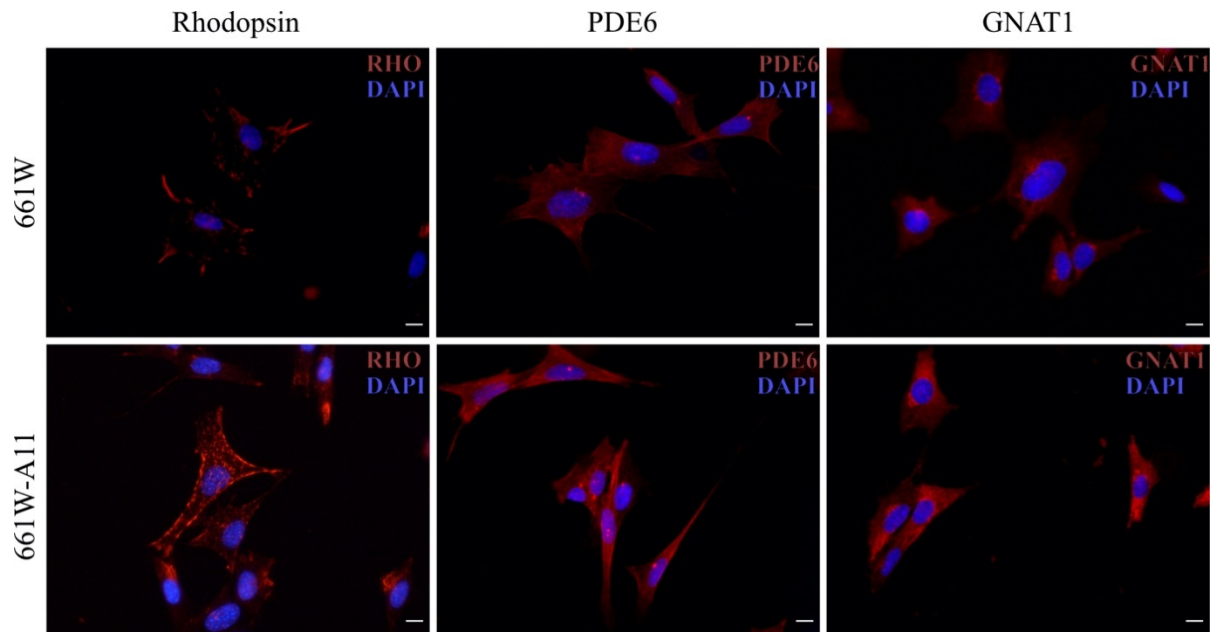


Figure 14. Immunofluorescence analysis confirmed a higher expression of rod photoreceptor-specific markers in 661W-A11 compared to 661W. Analyzed proteins RHO, PDE6B, and GNAT1 are stained in red, and nuclei are stained in blue (DAPI); Scale bar: 10 μ m.

4.1.3. Mimicking photoreceptor degeneration *in vitro*

The patients affected by the RP40 recessive form of RP bear biallelic mutations in *PDE6B* gene causing a loss of function of the PDE6 enzyme [369]. PDE6 mutation results in higher intracellular cGMP, calcium influx in photoreceptors, and activation of PKG and calpains [3]. We chose to treat 661W-A11 cells with zaprinast to mimic degeneration of photoreceptors in cells in culture. Zaprinast is a PDE inhibitor that almost specifically targets PDE6 and, to a limited extent, also PDE5 [370]. Different concentrations of zaprinast starting from 100 μ M to 500 μ M were used to treat 661W and 661W-A11 cells. Concentration-dependent decreased cell viability was observed in both cell types by MTT assay. The zaprinast treatment at concentrations 400 μ M or 500 μ M demonstrated a 25% cell viability reduction in 661W cells and a 30% reduction in 661W-A11 cells (Figure 15). For this reason, 400 μ M concentration was selected for cell viability experiments. Ethidium Homodimer staining was used to determine if reduced cell viability referred to increased cell death and not to interference with cell proliferation. We found that that 400 μ M zaprinast treatment for 24 hours caused 9% death in 661W cells and 14% cell death for 661W-A11 cells [371].

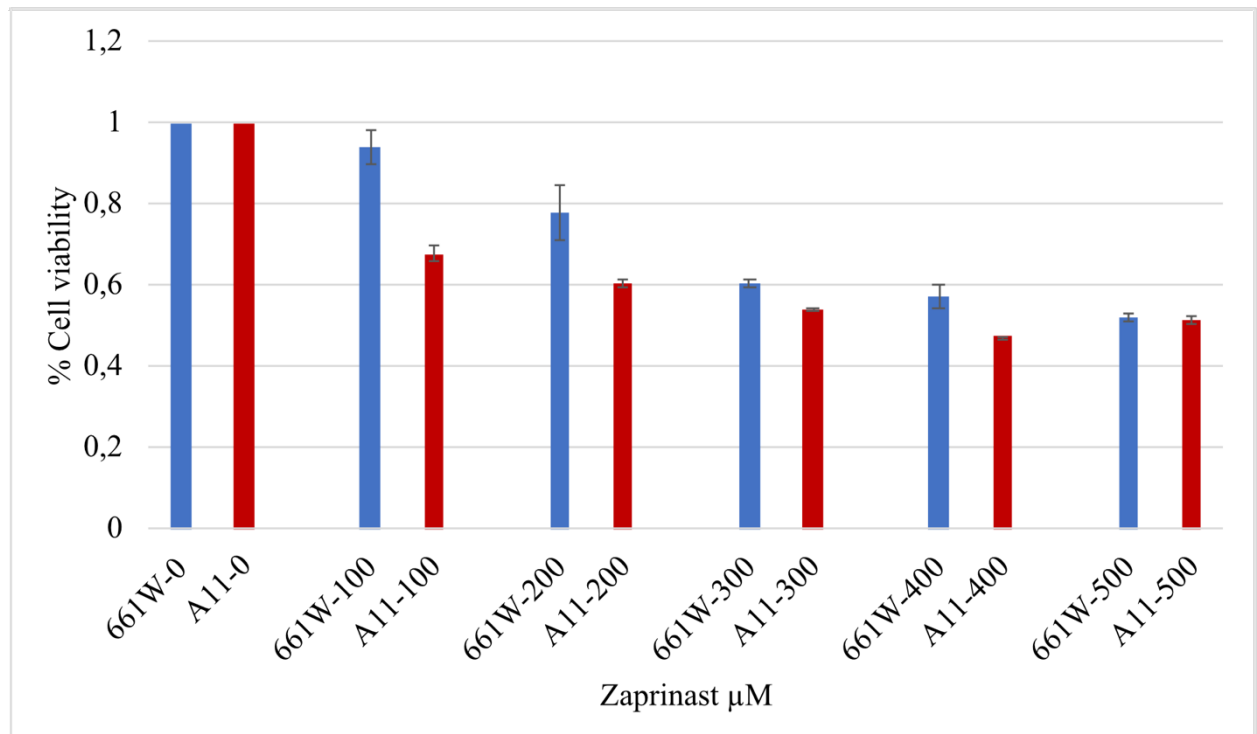


Figure 15. Effect of PDE6B inhibition by zaprinast treatment. Cell viability was evaluated by MTT assay in 661W-A11 (red) and 661W (blue) cells after scaling treatments with zaprinast for 24 hours. Values of cells treated with the vehicle (0 μM) were set as 100% cell viability.

A collaborator in my laboratory validated in 661W-A11 cells the molecular pathways activated as a consequence of PDE6 inhibition. Changes in cGMP and calcium levels were measured to show that zaprinast treatment could simulate loss of PDE6 function as in patients and related animal models. Specifically, a cGMP binding antibody was used to analyze cGMP levels and Fluo-4 staining to evaluate intracellular calcium. The inhibition of PDE6 with zaprinast showed a significant augmentation in intracellular calcium levels and cGMP [371].

Previous research found a link between elevated intracellular calcium and activation of calpain protease [36, 48, 68]. Therefore, calpain protease activity was assessed by measuring the cleavage of αII -spectrin, which is a calpain substrate. An increased amount of the 145–150 kDa fragments of αII -spectrin derived from calpain cleavage was observed in 661W-A11 cells after zaprinast treatment [371].

4.2. Identification of Gene Expression Changes During Photoreceptor Degeneration

Taking advantage of the newly generated 661W-A11 cell line, we designed an experiment to identify differentially expressed genes during photoreceptor degeneration. Secondly, we planned to identify transcripts that are affected by the treatment of the

previously characterized cGMP analogue CN03 [60]. 661W-A11 cells were stressed with the PDE6 inhibitor zaprinast for 22 hours and then co-treated with CN03 for 2 hours before collecting RNA. The choice of such a short time exposure to CN03 was based on the aim of identifying the most immediate response to the drug. For each sample, the same cells were cultured on slides and treated in parallel with cells collected for RNA preparation. The cells on slides were analyzed by ethidium homodimer staining to detect cell death rates and confirmed the toxic effect of zaprinast and the protective activity of CN03 even with such a short time of exposure to the cGMP analogue (Figure 16).

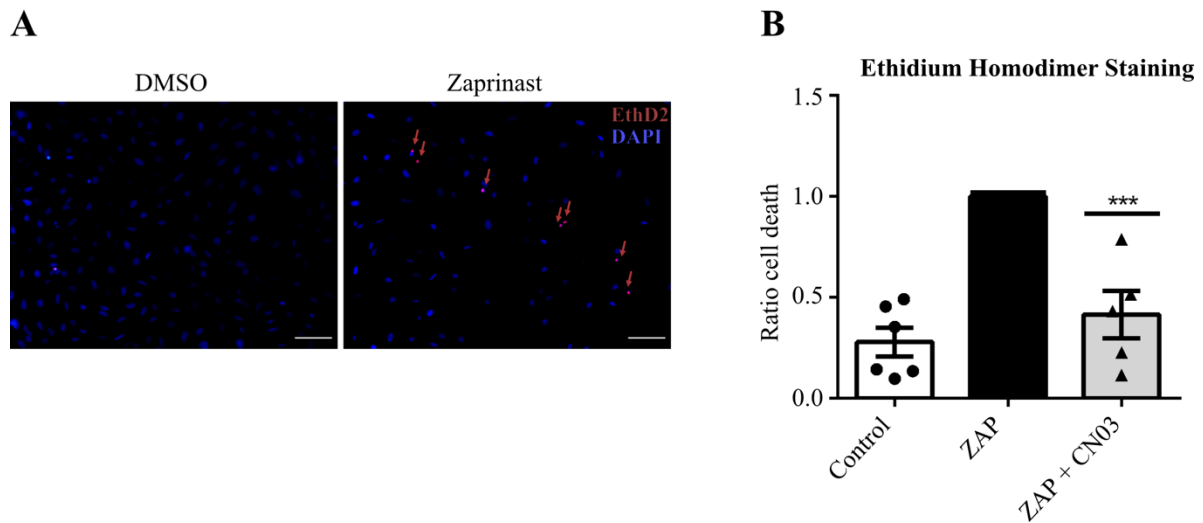


*Figure 16. Cell death rate of 661W-A11 cells treated with zaprinast (ZAP) or zaprinast in the presence of CN03 (ZAP+CN03). Ethidium homodimer staining analyses were performed after 24h of DMSO or zaprinast treatment at 400 μ M, cells were co-treated with CN03 at 50 μ M for 2h. (A) Fluorescent microscopy images of Ethidium homodimer (red) and DAPI (blue) staining of 661W-A11 cells treated with zaprinast or with vehicle (DMSO, control). Scale bar: 100 μ m Arrows indicate positive dying cells.. (B) Quantification of the results shown in panel A presented as percentage of cell death (defined as number of ethidium homodimer-stained cells divide by DAPI labeled cells). The value of cells treated with zaprinast was considered as 1. One-way ANOVA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.*

Four replicates for each condition (Control, zaprinast, and zaprinast+CN03) were used to commission an RNA-Seq analysis. The ROSALIND® software was used to analyze and visualize the results. For each group, replicates were clustered to visualize the similarity of samples within the RNA-Seq experiment with the software. Multidimensional scaling plot (Figure 17A) and Sample correlation map (Figure 17B) indicate a visual and graphical representation of the correlation between different samples, respectively. In general, replicates of each group showed similar patterns and clustered together, except for two outliers, one in the zaprinast group (EXP1) and a second one in the zaprinast+CN03 (EXP2) group. All the four samples from the control group (CTR1, blue dots in figure 17A) clustered together.

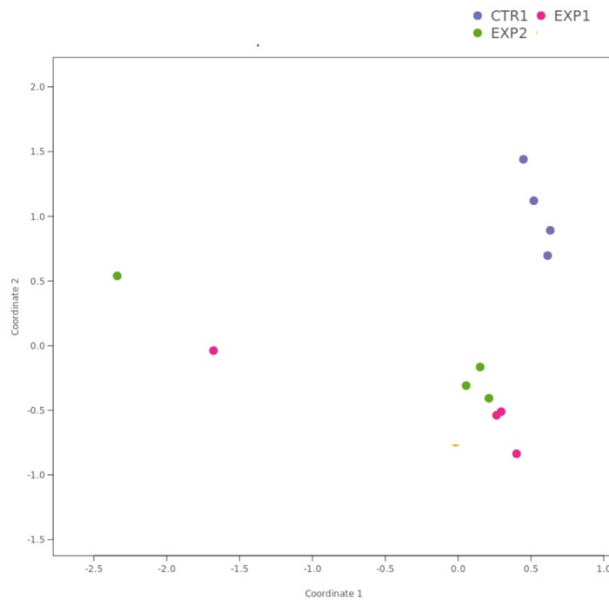
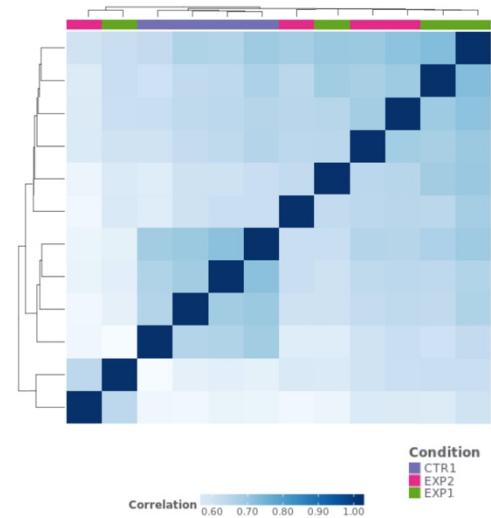
A**B**

Figure 17. Similarity of samples used in the RNA-Seq experiment. (A) Multidimensional scaling plot providing visual representation of the patterns of proximities among the different samples. (B) Sample correlation map providing a graphical representation of the correlation between different samples. The vehicle (DMSO) treated samples (CTR1) show similar patterns of expression and therefore cluster together and are strongly correlated. The zaprinast treated samples, with or without neuroprotective drug treatment (CN03), (EXP1 and 2) also cluster and correlate except for two outliers.

Based on this evidence, we initially focused our analysis on genes affected by increased cGMP and calcium, caused by zaprinast treatment. According to the results, 1312 genes were differentially expressed. Data were visualized with heatmap representation (Figure 18A) and by volcano plot graph, both of them indicating the up and down-regulated genes. The left-hand side of the dots in the volcano plot graph depicts the 697 down-regulated genes, and the right-hand side of the volcano plot dots in the graph shows the 615 up-regulated genes (Figure 18B).

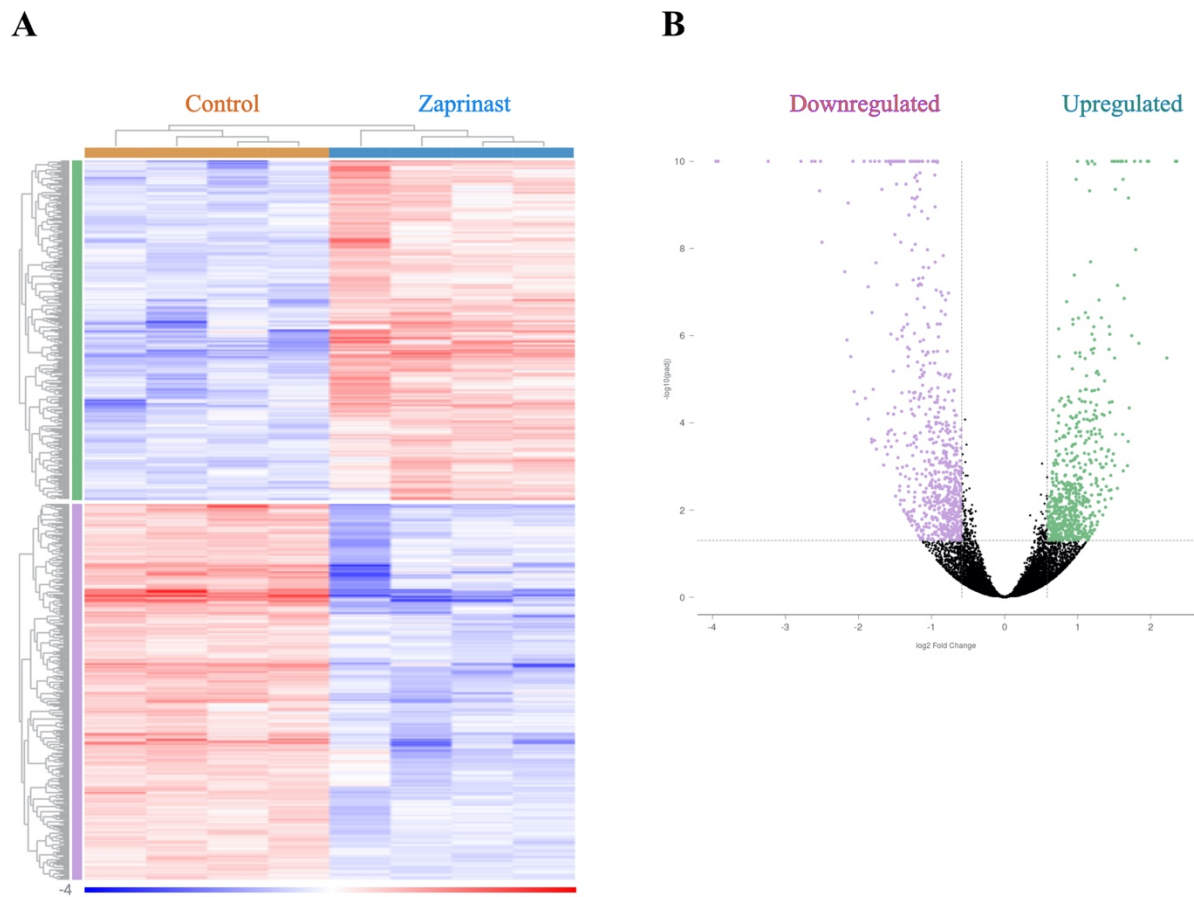


Figure 18. Differentially expressed genes comparing control (DMSO) and zaprinast treated samples. (A) Sample correlation heatmap showing gene expression changes of differentially expressed genes in control vs zaprinast treated groups. (B) Volcano plot showing the differentially expressed genes with each dot representing a gene within the performed comparison. Genes that are upregulated after zaprinast treatment are shown in green, downregulated genes are shown in violet.

Even if the number of up and down-regulated genes were close to each other, when we analyzed fold changes, the most affected genes were generally down-regulated. A list with the most upregulated and the most downregulated genes is presented in Table 5. Genes that were subsequently chosen as candidate genes are highlighted in orange or blue if upregulated or downregulated, respectively.

Table 5. Most upregulated and the most downregulated genes from RNA-Seq analysis.

Upregulated				
Name	Fold Change	p-Value	p-Adj	Description
<i>Ypel3</i>	5,12801	8,54E-25	8,19E-22	Yippee Like 3
<i>Cdkn1a</i>	5,06545	2,15E-21	1,40E-18	Cyclin Dependent Kinase Inhibitor 1A
<i>Angptl4</i>	4,66291	3,97E-08	3,24E-06	Angiopoietin Like 4
<i>Klf2</i>	3,92575	8,87E-23	7,09E-20	Kruppel Like Factor 2
<i>Cirbp</i>	3,90353	1,62E-15	5,69E-13	Cold Inducible RNA Binding Protein
<i>Gdf15</i>	3,87918	7,95E-24	7,16E-21	Growth differentiation factor 15
<i>Dhrs7</i>	3,63463	1,82E-21	1,25E-18	Dehydrogenase/Reductase 7
<i>Ypel1</i>	3,57747	1,61E-08	1,49E-06	Yippee Like 1
<i>Atf3</i>	3,47037	6,83E-11	1,06E-08	Activating Transcription Factor 3
<i>Mt1</i>	3,43424	1,60E-26	1,78E-23	Metallothionein 1A
<i>Cyp4f13</i>	3,34343	1,03E-08	9,99E-07	Cytochrome P450 CYP4F13
<i>Txnip</i>	3,2661	8,09E-07	4,55E-05	Thioredoxin Interacting Protein
<i>Polr3gl</i>	3,24132	3,86E-12	6,93E-10	RNA Polymerase III Subunit GL
<i>Zc3h6</i>	3,23297	6,86E-06	0,000267	Zinc Finger CCCH-Type Containing 6

Downregulated				
Name	Fold Change	p-Value	p-Adj	Description
<i>Postn</i>	-15,4878	2,64E-35	5,43E-32	Periostin
<i>Il1rl1</i>	-15,1698	0	0	Interleukin 1 Receptor Like 1
<i>Colla1</i>	-9,42982	5,56E-39	1,33E-35	Collagen Type I Alpha 1 Chain
<i>Acta2</i>	-6,90844	0	0	Actin Alpha 2
<i>Colla2</i>	-6,235	0	0	Collagen Type I Alpha 2 Chain
<i>Medag</i>	-6,04197	1,09E-15	4,03E-13	Mesenteric Estrogen Dependent Adipogenesis
<i>Kif26b</i>	-5,78385	2,54E-12	4,75E-10	Kinesin Family Member 26B
<i>Igf1</i>	-5,73554	4,19E-39	1,21E-35	Insulin growth factor 1
<i>Tagln</i>	-5,65874	4,54E-11	7,18E-09	Transgelin
<i>Col27a1</i>	-4,55692	2,42E-10	3,41E-08	Collagen Type XXVII Alpha 1 Chain
<i>Il1rn</i>	-4,46367	1,34E-08	1,26E-06	Interleukin 1 Receptor Antagonist
<i>Trac</i>	-4,41373	5,20E-12	9,02E-10	T Cell Receptor Alpha Constant
<i>Hist1h4b</i>	-4,30392	3,65E-08	3,02E-06	H4 Clustered Histone 2
<i>Slc39a10</i>	-4,21879	4,87E-15	1,67E-12	Solute Carrier Family 39 Member 10
<i>Wnt7b</i>	-4,1681	2,88E-07	1,92E-05	Wnt Family Member 7B
<i>Hist1h2ag</i>	-4,05314	6,33E-07	3,67E-05	Histone H2A type 1
<i>Ppal</i>	-3,79484	4,50E-41	1,62E-37	Inorganic Pyrophosphatase 1
<i>Axin2</i>	-3,74449	4,38E-07	2,73E-05	Axin 2
<i>Chodl</i>	-3,65198	5,90E-10	7,51E-08	Chondrolectin

In order to retrieve information on common molecular aspects of the genes differentially expressed upon block of the PDE6 enzyme in a rod-like photoreceptor cells, RNA-Seq data were analyzed by several software. We first looked whether we could

associate our data to ocular diseases. Jensen Diseases retrieved as first hit “glaucoma” correlating to this disease upregulation of *Tgfr3* and downregulation of *Cav1*, *Lox11*, *Dclk1*, *Six1*, *Mtap*, *Col11a1*, *Col5a1* (Figure 19). Glaucoma was also highlighted by ClinVar, which associated to this disease upregulation of *Optn* and downregulation of *Wdr36*.

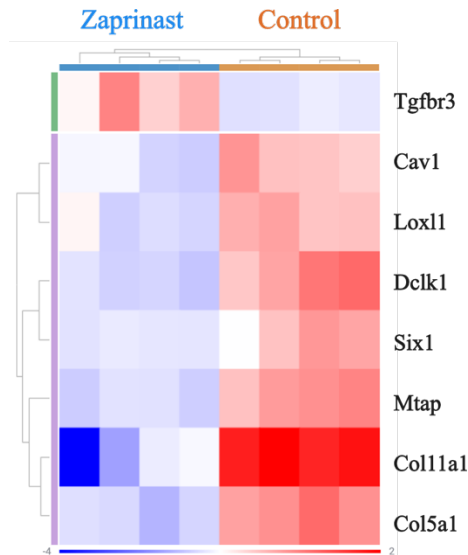


Figure 19. Sample correlation heatmap showing glaucoma correlated genes identified by Jensen Diseases.

The analyses on pathways significantly affected by the block of PDE6, identified cholesterol biosynthesis as a metabolic pathway affected by the treatment. The cholesterol biosynthesis pathway was, in fact, highlighted by WikiPathways, BioPlanet, PANTHER, REACTOME and BIOCYC. Interestingly, several enzymes of the cholesterol biosynthesis pathways appeared downregulated in zaprinast treated cells. Only one gene was found upregulated and was *Acot2*.

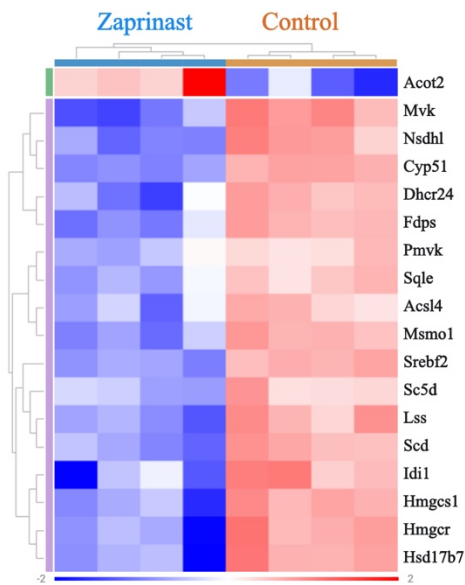
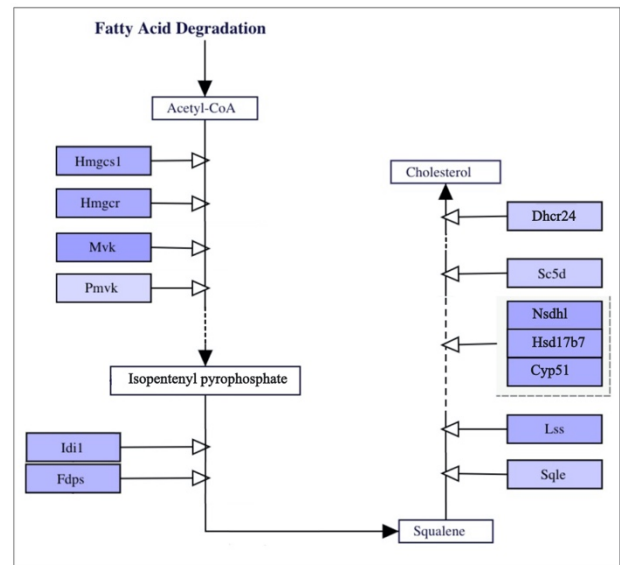
A**B**

Figure 20. Cholesterol-related genes are differentially expressed genes in the comparison between DMSO (control) and zaprinast treated samples. (A) Sample correlation heatmap showing gene expression changes of cholesterol-related genes. Many genes involved in cholesterol biosynthesis are downregulated after zaprinast treatment. (B) Schematic representation of the biochemical pathway from acetyl-CoA to cholesterol in which differentially expressed cholesterol genes are shown in blue.

The mainly affected cholesterol-related genes are visualized and listed using the heatmap analysis in Figure 20A. The pathway analysis tool, Wikipathway, was used to visualize their level of involvement in cholesterol biosynthesis (Figure 20B). The cholesterol biosynthesis and metabolism pathway, known as the mevalonate pathway, is controlled by several major enzymes to synthesize the cholesterol in the cells, such as Hmgcs1 and Hmgcr, Mvk, Pmvk, Id1, and Fdps. All the genes found downregulated in Zaprinst stressed 661W-A11 cells encode the enzymes necessary for synthesizing the squalene, direct intermediate product of cholesterol (Figure 20B). *Sqle*, *Lss*, *Sc5d*, *Dhcr24* genes, and implicitly *Nsdhl*, *Hsd17b7*, *Cyp51* genes, encode enzymes that take part in the cholesterol biosynthesis after squalene synthesis (Figure 20B). Interestingly, the master regulator of sterol and fatty acid synthesis, transcription factor *Sreb2*, was also found downregulated. In contrast to all those genes, only a gene was up-regulated, *Acot2*, encoding an enzyme which hydrolyzes acyl-CoAs to the free fatty acid and coenzyme A.

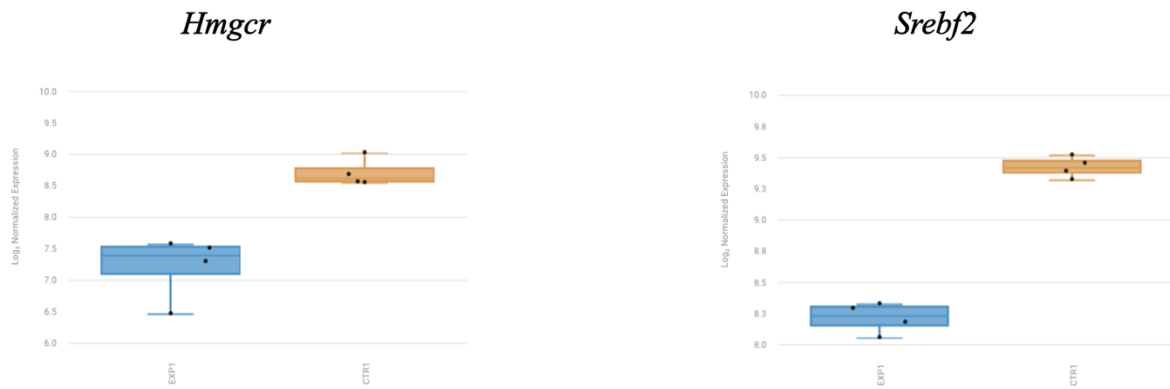


Figure 21. Identification of downregulated genes. The Barplots show the normalized gene expression in 661W-A11 cells after zaprinast treatment (EXP1) in blue and control (CTR1) in orange for two representative genes, *Hmgcr* on the left-hand side and *Srebf2* on the right-hand side.

We selected a list of genes for further validation. Figure 21 presents, as examples, the histograms of the expression of two of the selected genes that were downregulated after zaprinast treatment. We classified the candidate genes into two groups, as reported in Table 6; "cholesterol-related genes" and "general genes". Primers for detecting these genes were designed, and Real-time qPCR was performed on RNA extracted from 661W-A11 cells or murine retinas.

Table 6. Candidate genes chosen from RNA-Seq data analysis

Classification		Gene symbols	Gene names
Cholesterol-related	Up	<i>Acot2</i>	Acyl-CoA Thioesterase 2
		<i>Abca1</i>	ATP-binding cassette transporter 1
		<i>Abca2</i>	ATP-binding cassette transporter 2
	Down	<i>Srebf2</i>	Sterol Regulatory Element Binding Transcription Factor 2
		<i>Hmgcr</i>	3-Hydroxy-3-Methylglutaryl-CoA Reductase
		<i>Mvk</i>	Mevalonate Kinase
		<i>Nsdhl</i>	NAD(P) Dependent Steroid Dehydrogenase-Like
		<i>Fdps</i>	Farnesyl Diphosphate Synthase
General	Up	<i>Ypel3</i>	Yippee Like 3
		<i>Cdkn1a</i>	Cyclin Dependent Kinase Inhibitor 1A
		<i>Hdac5</i>	Histone Deacetylase 5
	Down	<i>Postn</i>	Periostin
		<i>Il1rl1</i>	Interleukin 1 Receptor Like 1
		<i>Igf1</i>	Insulin Like Growth Factor 1
		<i>Nfatc1</i>	Nuclear Factor Of Activated T Cells 1
		<i>Hk2</i>	Hexokinase 2
		<i>Axin2</i>	Axin2

4.2.1. Validation of candidate genes on 661W-A11 cell line

We proceeded with the confirmation by Real-time qPCR of some of the differentially expressed genes in samples from 661W-A11 cells. We chose the most affected genes mentioned above related to cholesterol biosynthesis. Moreover, *Abca1* and *Abca2*, that encode for cholesterol transporters, were also included in the list of cholesterol-related genes, because found upregulated, even if not indicated by the pathways analysis tools.

The calculation of the relative gene expression confirmed most of the genes identified by the RNA-Seq data. Otherwise, *Abca1*, *Abca2*, *Acot2*, all upregulated cholesterol-related genes in the RNA-Seq data were found highly downregulated. The other cholesterol-related down-regulated genes, *Srebf2*, *Hmgcr*, *Mvk*, *Nsdhl*, *Fdps*, were all confirmed in 661W-A11

cells upon zaprinast stress. Higher levels of *Hdac5* in zaprinast treated cells could not to be confirmed in new samples of 661W-A11 cells (Figure 22).

The genes that were confirmed the RNA-Seq gene expression results by Real-time qPCR were chosen for further analysis on the murine retinas.

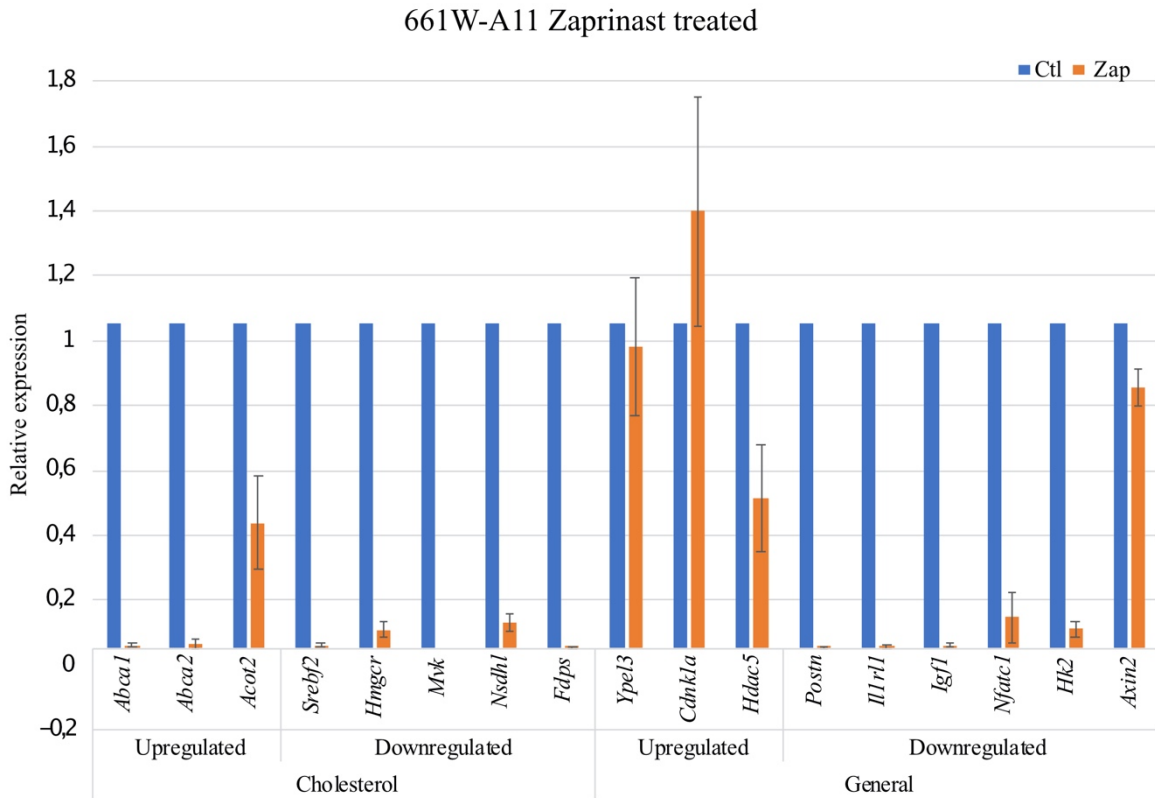


Figure 22. Relative expression analysis by Real-time qPCR of candidate genes chosen from the differentially expressed genes from the RNA-Seq analysis. Gene expression of 661W-A11 cells treated with zaprinast (orange) was compared to the control (blue) by the delta-delta Ct method. The analysis confirmed the results of the RNA-Seq for most of the genes.

4.2.2. Validation of Differentially Expressed Genes on Animal Models of RP

To assess the biological significance of the RNA-Seq results *in vivo*, Real-time qPCR was performed for the candidate genes on RNA extracted from retinas of mouse models of RP and compared to relevant, age-matched wild type retinas. The *rd1* and *rd10* mice carry a mutation in the PDE6B subunit and these are the closest murine models to the 661W-A11 cell system, which was based on PDE6 inhibition. We collected RNA at PN11 for *rd1* and PN18 for *rd10*. The *Rho*^{P23H/+} knock-in mouse, is characterized by a dominant mutation in RHO and shares with *rd1* mutants the intracellular increase of calcium. We chose PN19 for the analysis being the age with the peak of cell death [58].

We collected RNA from 2 or 3 biological replicates for mutant animal and from age-matched wild type animals.

First, the relative expression of cholesterol-related genes was analyzed in the 3 animal models. Data from *rd1* mutant samples did not confirm any of the expression changes found *in vitro*. The study on *rd10* otherwise, while with some variability, overall confirmed the downregulation of genes encoding for enzymes of the cholesterol pathway. Among these, statistical analysis highlighted *Srebf2*, *Hmgcr* and *Fdps* as significantly downregulated in the mutant animals. Moreover, *Abca2*, which was observed upregulated by RNA-Seq but downregulated by Real-time qPCR in the *in vitro* model, appeared to be significantly downregulated in *rd10* mutant animals. In the *Rho*^{P23H/+} instead, *Srebf2* and *Hmgcr* genes were found to be significantly upregulated in the mutant animals, while *Nsdhl* was the only cholesterol-related gene that confirmed to be downregulated.

Furthermore, some of the other candidate genes that we analyzed were not confirmed, but *Axin2* is confirmed downregulated in the *rd10* animals, *Postn* in *Rho*^{P23H/+}, and *Hk2* in both models. The results of the Real-time PCR analysis are shown in figure 23.

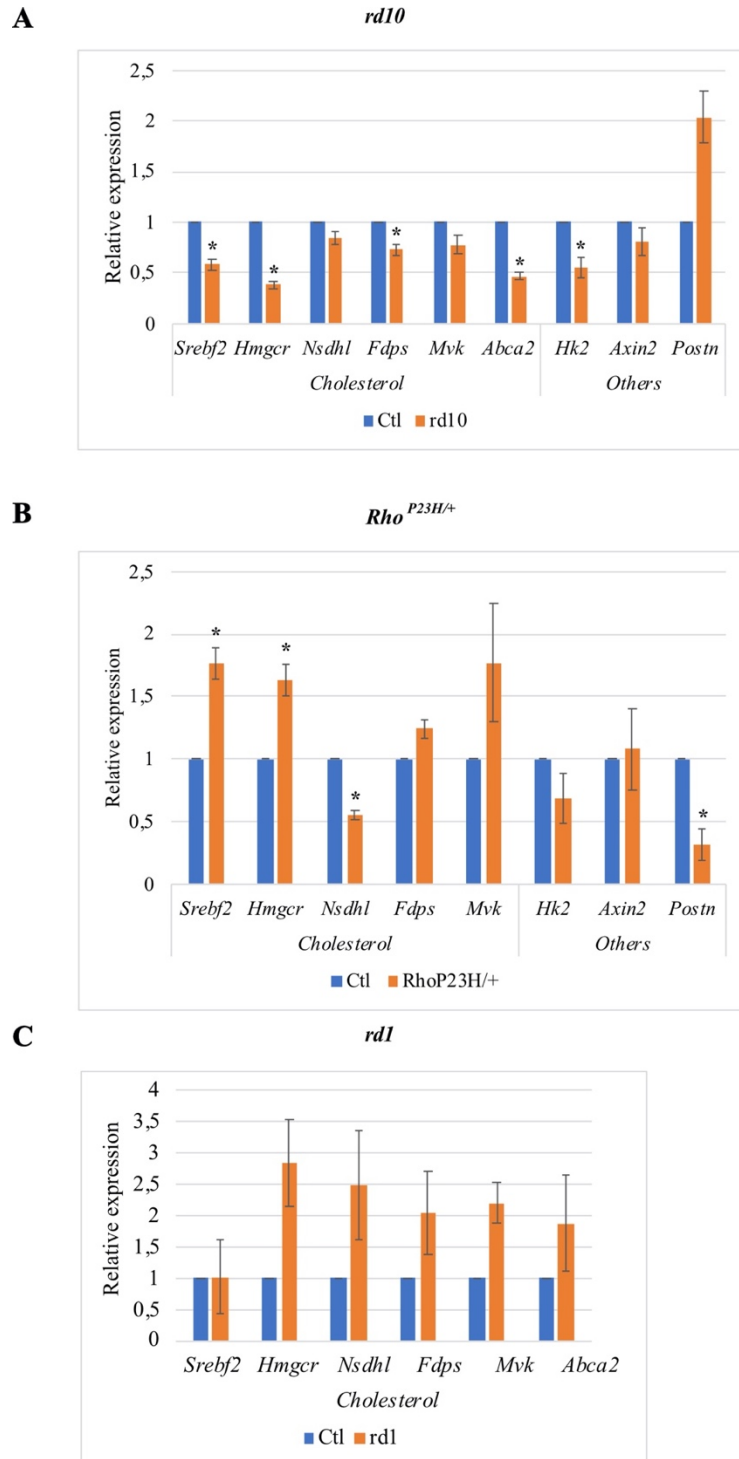


Figure 23. Relative expression analysis by Real-time qPCR of candidate genes in animal models. Mutant animals (orange) were compared to age and background matched controls (blue) by the delta-delta Ct method. (A) The analysis on *rd10* mutant retinas confirmed the downregulation of genes encoding for enzymes of the cholesterol pathway and in particular of *Srebf2* and *Hmgcr*. Downregulation of *Hk2* and *Axin2* genes was confirmed. (B) In *Rho^{P23H/+}* mutant cholesterol-related genes appeared upregulated except for *Nsdhl*. From the group of the general genes, the downregulation of *Postn* was confirmed. (C) The *rd1* mutants did not confirm any downregulation of the cholesterol-related genes.

With the aim of identifying targets of the cGMP analogue CN03, which showed a protective effect *in vitro*, as previously demonstrated in the *rd10* mouse model *in vivo* [84],

we analyzed the RNA-Seq data derived from the comparison of 661W-A11 cells treated with zaprinast and 661W cells exposed to CN03 during the zaprinast stress. The analysis did not retrieve significant changes in gene expression applying the adjusted p-value parameter. When we performed the analysis with a less stringent parameter, which is p-value without adjustment, and we could find 50 genes affected by the CN03 treatment, 20 of these are reported in table 7. Interestingly, the CN03 treatment retrieved mostly upregulated genes (*i.e.* 32 genes) and less downregulated (*i.e.* 18 genes).

Table 7. 10 most upregulated and 10 most downregulated genes in cells treated for 2 hours with CN03 during the zaprinast stress compared with zaprinast treated cells.

Upregulated				
Name	Fold Change	p-Value	p-Adj	Description
<i>Murc</i>	1,80694	3,73E-05	0,13251	Muscle-Restricted Coiled-Coil Protein
<i>Ackr3</i>	1,73528	0,00016	0,32076	Atypical Chemokine Receptor 3
<i>Wisp1</i>	1,71215	6,41E-05	0,18211	WNT1-inducible-signaling pathway protein 1
<i>Ogt</i>	1,70898	0,00028	0,42722	O-Linked N-Acetylglucosamine Transferase
<i>Il1rl1</i>	1,70110	0,00045	0,49535	Interleukin 1 Receptor Like 1
<i>Cd248</i>	1,67696	0,00032	0,42722	CD248 Molecule
<i>Aqp5</i>	1,66247	0,00067	0,55600	Aquaporin 5
<i>Runx3</i>	1,64925	2,49E-05	0,13251	RUNX Family Transcription Factor 3
<i>JmJd4</i>	1,62853	0,00105	0,70865	Jumonji Domain Containing 4
<i>Trac</i>	1,62228	0,00139	0,82623	T Cell Receptor Alpha Constant
<i>Tgfb2</i>	1,60218	2,61E-05	0,13251	Transforming Growth Factor Beta 2

Downregulated				
Name	Fold Change	p-Value	p-Adj	Description
<i>Lonrf1</i>	-1,75088	2,96E-05	0,13251	LON Peptidase N-Terminal Domain And Ring Finger 1
<i>Cyr61</i>	-1,71196	0,00032	0,42722	Cysteine-rich angiogenic inducer 61
<i>Mxd1</i>	-1,67586	0,00037	0,44077	MAX Dimerization Protein 1
<i>C2cd2l</i>	-1,62964	0,00124	0,80044	C2CD2 Like
<i>Ccsap</i>	-1,57932	0,00255	0,95497	Centriole, Cilia And Spindle Associated Protein
<i>Znrf3</i>	-1,57473	0,00248	0,95497	Zinc And Ring Finger 3
<i>Sesn2</i>	-1,56556	0,00251	0,95497	Sestrin 2
<i>Slc25a32</i>	-1,55514	0,00054	0,49899	Solute Carrier Family 25 Member 32
<i>Etv5</i>	-1,55026	0,00274	0,97231	ETS Variant Transcription Factor 5
<i>Map3k13</i>	-1,54135	0,00301	0,99985	Mitogen-Activated Protein Kinase Kinase Kinase 13

4.3. *In vitro* Differentiation of 661W-A11 Cells

4.3.1. Identifying differentiation conditions for 661W-A11 cells

The availability *in vitro* of mature rod-like cells could help us to mimic retinal degeneration and test therapeutic compounds. While the genetically modified 661W-A11 cells demonstrated to reproduce *in vitro* some of the features of a rod photoreceptor, the cell cycle is still active in these cells while one of the characteristics of neurons is to exit the cell cycle and be postmitotic cells. Therefore, *in vitro* differentiation of 661W-A11 cells could result in cells acquiring a phenotype closer to rod photoreceptors.

To this aim, we tested four different culture media on 661W and 661W-A11 cells (recipes are reported in the Material and Methods section 3). The differences among differentiation media are mostly related to the type of culture media (DMEM or DMEM-F12), amount of FBS, and containing or not containing N2. The other differentiation components, previously developed by our lab for retinal stem cells differentiation, including bFGF, taurine, heparin, retinoic acid and sodium butyrate, were maintained unaltered in all media recipes [358].

We proceeded by defining differentiation conditions for 661W and 661W-A11 cells by analyzing rhodopsin expression by immunofluorescence. After three days of differentiation, we observed that substituting DMEM with DMEM-F12 allowed 661W and 661W-A11 cells to change shape and form protrusions. We noticed that the improvement in rhodopsin content was very limited in the 661W cell line but very evident in the 661W-A11 line. Specifically, differentiation medium 4 (DM4), prepared with DMEM-F12 culture medium, without FBS but containing bFGF, taurine, heparin, retinoic acid, sodium butyrate and N2, allowed the 661W-A11 cells to acquire an elongated neuronal shape and increased rhodopsin, based on immunofluorescence staining. Otherwise, Pde6 staining did not significantly change (Figure 24A and 24B).

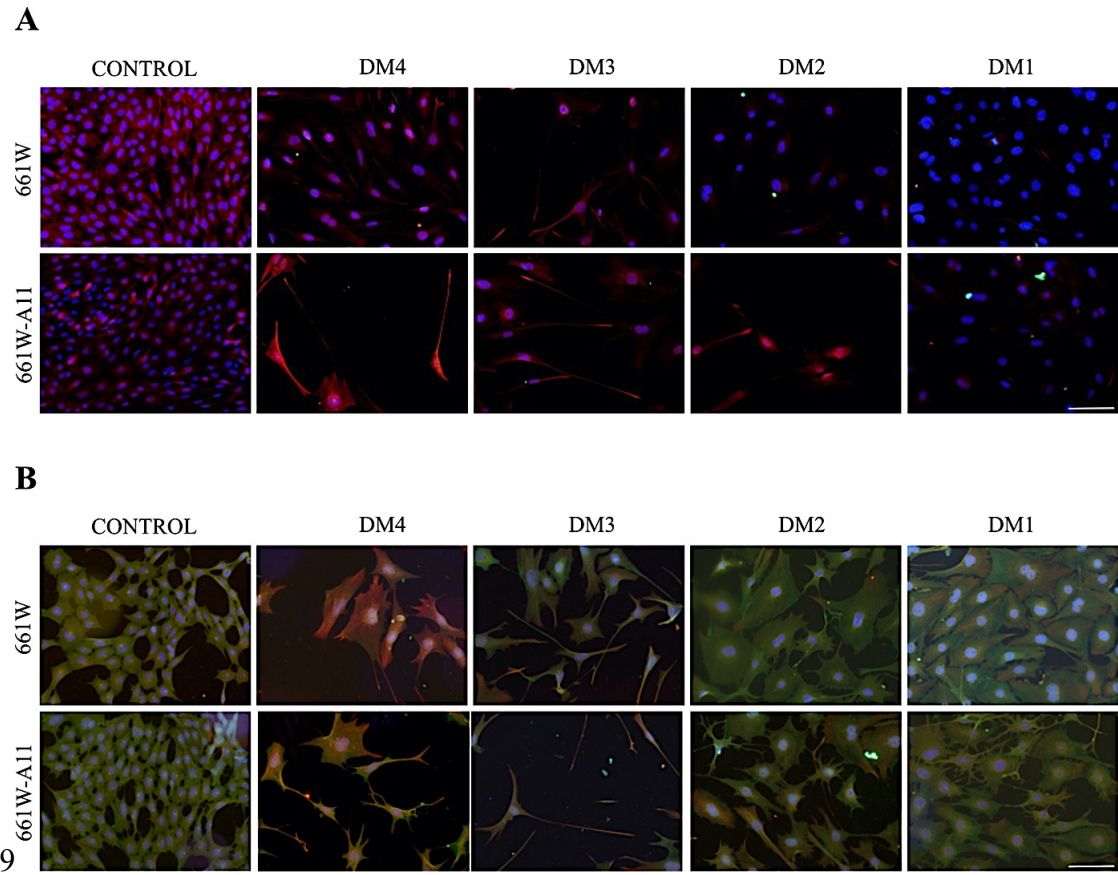


Figure 24. Immunofluorescence analysis comparing four differentiation media on 661W and 661W-A11 cells cultured for 3 days. (A) Rhodopsin (1D4) is stained in red and nuclei in blue (DAPI). (B) Rhodopsin (1D4 monoclonal antibody) is in red, Pde6b in green, and nuclei in blue (DAPI). Scale bar: 100 μ m. Control (culture) medium: DMEM + 10%FBS; DM1: DMEM + bFGF, taurine, heparin, retinoic acid, sodium butyrate; DM2: DMEM + 10% FBS, N2, bFGF, taurine, heparin, retinoic acid, sodium butyrate; DM3: DMEM-F12 + 1% FBS, N2, bFGF, taurine, heparin, retinoic acid, sodium butyrate; DM4: DMEM-F12 + N2, bFGF, taurine, heparin, retinoic acid, sodium butyrate.

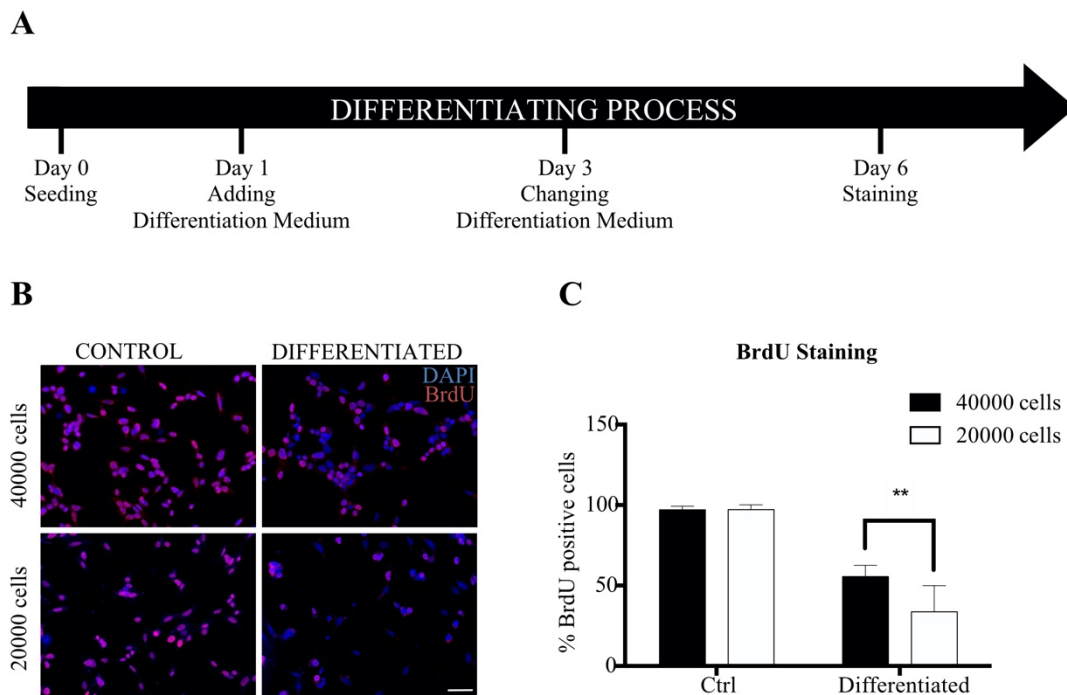


Figure 25. BrdU staining assay to assess exit from the cell cycle of 661W-A11 cells. (A) Workflow of the differentiation procedure. (B) Fluorescence microscopy images of BrdU positive 661W-A11 (control) and differentiated 661W-A11 cells. (C) Histogram from data shown in panel B showing percentage of BrdU positive cells based on its ratio to total number of cells counted by DAPI labeling. BrdU is shown in red and nuclei are stained in blue (DAPI). Control groups (Ctrl) showed 97.10% and 97.03% BrdU positive cells, respectively. BrdU positive cells significantly decreased in differentiated cells, and the percentage was 55.50% and 33.56%, respectively.

For effective differentiation, besides time of exposure to the differentiation medium, the percentage of confluency of the cells and time of differentiation were found to highly influence the elongation of the cells. We, thus, tested different numbers of cells at seeding time, from 5000 to 100000 in each well of a 24 wells plate, and different times of exposure to differentiation media, from 3 to 7 days (Figure 25A). As read-out of this experiment, we decided to evaluate exit from the cell cycle determined by decline of BrdU staining. BrdU is a modified nucleotide that can be integrated into the DNA during the S phase and can be visualized by immunofluorescence. In the first experiments we found that 3 days of differentiation were not sufficient for the cells to exit the cell cycle. Seven days, otherwise, were a time when cells started to be not so healthy (data not shown). We chose 5 days for our statistical analyses. We compared 661W-A11 and five days differentiated 661W-A11 seeded at two different amounts of cells, 20000 and 40000 cells. The count of the percentage of cycling cells, based on BrdU labelled cells showed that the differentiation procedure decreased the number of BrdU labelled cells and that the decrease was more pronounced when 20000 cells were seeded (33.56% labelled with a decrease of 66.44%), than when

40000 cells were seeded (55.50% labelled with a decrease of 44.50%) (Figure 25B and C). Our data indicated that 20000 cells per well were the optimal condition for 661W-A11 to differentiate and become post-mitotic.

After defining the differentiation conditions, we compared differentiated and not differentiated 661W-A11 cells on rod photoreceptor-specific protein expression by immunofluorescence assay. We observed that the most evident change was the shape of the 661W-A11 cells after five days of differentiation. Most of the cells acquired a neuronal phenotype and their elongated bodies and filaments were quite visible. In contrast, the control cells were almost spherical and smaller than the differentiated cells. We could detect Rhodopsin kinase, GNAT, Peripherin, RHO and PDE6B proteins. After differentiation, rhodopsin kinase predominantly localized around the cell membrane and especially at the tip of the filaments. GNAT also localized at the tip of the filaments, partially co-localizing with Rhodopsin kinase. GNAT is also visible around the nucleus as green spots (Figure 26A). Peripherin expression seemed not to increase after differentiation, but it accumulated in the filaments of the cells (Figure 26B). For Rhodopsin and PDE6, differentiated cells had a stronger signal for both proteins, indicating that their expression could be increased by differentiation (Figure 26C). When we compared the controls and differentiated cells, before differentiation rhodopsin could be detected in the cell bodies with a stronger signal along the membrane. After differentiation it appears to be well-distributed along the filaments of the cells and in the central part of the cell bodies. PDE6 is distributed in all the cell bodies with the signal being stronger at the cell membrane. After differentiation the distribution remains similar, but the intensity of the signal appears increased.

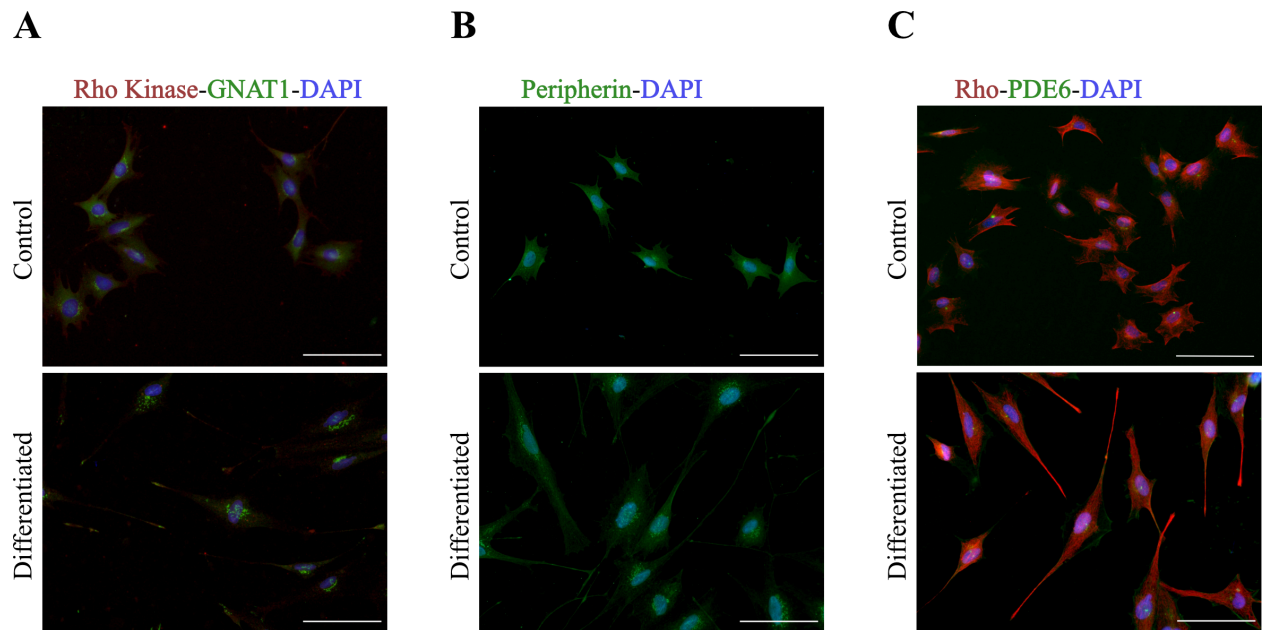


Figure 26. Immunofluorescence analysis of Rod photoreceptor-specific markers on 661W-A11 cells not differentiated (control) and differentiated for 5 days with DM4. (A) Analysis of Rhodopsin (Rho) kinase (red) and GNAT1 (green). (B) Analysis of Peripherin (green). (C) Analysis of Rhodopsin (Rho) (red) and PDE6 (green). Nuclei were stained in blue (DAPI). Scale bar: 100 μ m.

Our data indicated that five days of treatment with differentiation medium DM4 resulted in exit of about 70% of the cells from the cell cycle, changes of protein distribution inside the cells and possible increased expression of some rod-specific-proteins.

The not complete exit from the cell cycle of the 661W-A11 cells, prompted us to evaluate treatment with drugs to interfere with DNA replication. Hence, we designed an experiment with aphidicolin treatment to allow a more uniform differentiation of 661W-A11 cells. Aphidicolin is an antibiotic that acts as a reversible inhibitor of DNA polymerase. Previous studies showed that aphidicolin could block the cell cycle at the early S phase, which provides a homogeneous cell condition for starting the differentiation process [372]. Moreover, another study indicated that aphidicolin also improved the elongation of cells that acquired neuronal cells shapes [372].

Based on the information from the literature, we planned two experimental designs. Firstly, to determine the applicable concentration to block cell proliferation, we treated 661W-A11 cells with different concentrations of aphidicolin for 16 hours and observed the cells before and after aphidicolin treatment (Figure 27A). Based on light microscopy images, we defined that the cells treated with 0.05 μ M, 0.1 μ M, 0.3 μ M looked healthy. In contrast, a lot of cells treated with 1 μ M aphidicolin were floating and detached from the plate and this concentration was considered toxic and not used for further experiments (Figure 27A).

Secondly, we designed an experiment to test cell viability including two data sets: 1) 661W-A11 cells were treated for 16 hours with aphidicolin at 0.05 μ M, 0.1 μ M, 0.3 μ M concentrations and aphidicolin was removed before starting the five days of differentiation; 2) 661W-A11 cells were treated for 16 hours with aphidicolin at 0.05 μ M, 0.1 μ M, 0.3 μ M concentrations and aphidicolin was maintained during the five days of differentiation (Figure 27B).

We found that the effect of aphidicolin did not significantly affect cell viability at all tested concentrations and the maintenance or not during differentiation was also not toxic.

Nevertheless, we noticed detached cells in both data sets 16 hours after aphidicolin treatment, when we added differentiation media. Worried that aphidicolin could strongly affect cell metabolism and physiology, we decided not to continue to treat the cells with aphidicolin based on these findings.

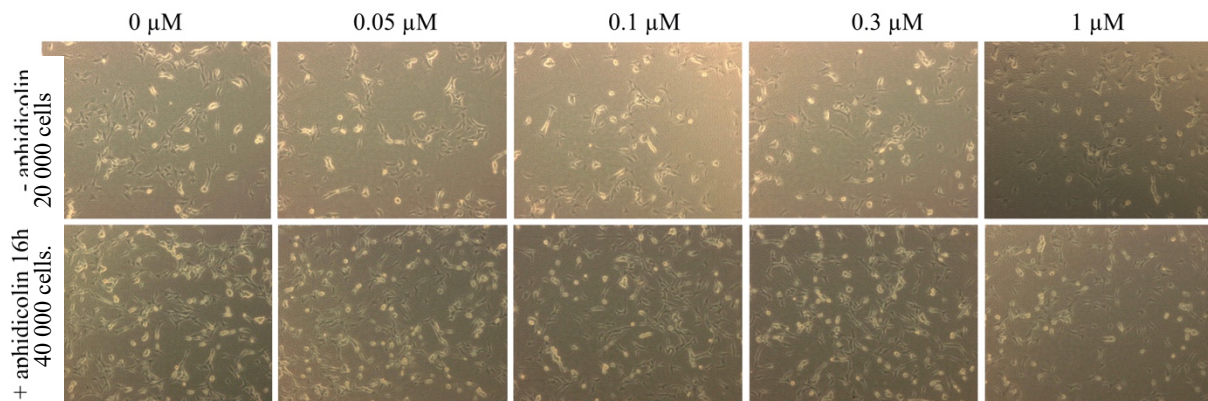
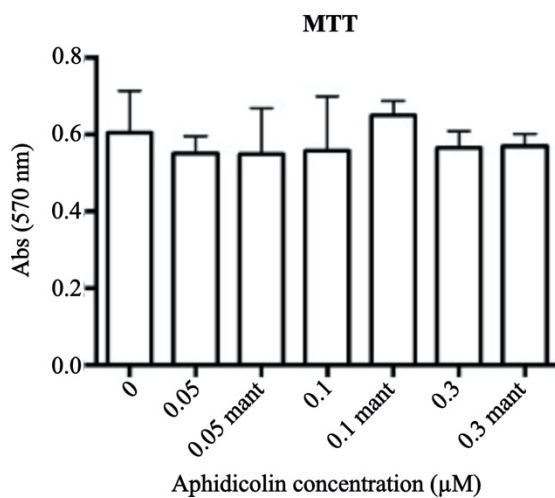
A**B**

Figure 27. Aphidicolin effects on 661W-A11 cells. (A) Light microscopy images of the cells plated at 20,000 and 40,000 cells per. Pictures of the wells were taken to compare the morphology of the cells after 16 hours aphidicolin treatments concentrations at 0 μ M, 0.05 μ M, 0.1 μ M, 0.3 μ M, and 1 μ M. (B) Cell viability analysis of aphidicolin treated cells by MTT assay. (B) Cell viability MTT analysis on cells treated with different concentrations of aphidicolin either added 16h before differentiation or maintained during differentiation (mant).

Many factors can have an impact on *in vitro* differentiation of the cells. One of the factors can be the substrate on which you culture the cells, that significantly influences the 2D cell culture. Coating the plate surface with different matrix protein is commonly used to grow primary cells. While the 661W cells could be easily cultured on plastic or glass surfaces, 661W-A11 cells appeared more sensitive than the original cell line. To improve differentiation of 661W-A11 cells we decided to test laminin, poly-D-Lysine and extracellular matrix (ECM) (derived from a mouse tumor) as coating surfaces. When the cells were grown on the polylysine or ECM coated surfaces, we did not observe improvement in the cell culture and differentiation (data not shown). In contrast, the laminin-

coated surface showed a stable and improved cell culture condition. Cells looked healthier and formed a lot of filaments with a higher elongation efficiency (Figure 28).

Many types of recombinant laminins exist for cell culture studies. Laminin-521 (LN521) was reported by the selling company to provide a stable environment for embryonic stem cells and robust differentiation for induced pluripotent stem cells. Investigating 661W-A11 cells differentiation, we compared regular laminin with the LN521 coated coverslips. After five days of differentiation, we analyzed rod photoreceptor-specific protein expression by immunofluorescence analysis.

We observed that cell differentiated on LN521 resulted in strong expression of rod-specific proteins (Figure 28A-C). The feature that showed the best improvement when cells were cultured on LN521 was the staining of acetylated-alpha-tubulin, a marker for tubulin in the connecting cilia. We found that most elongated filaments were positive for acetylated-alpha-tubulin only when cells were differentiated on LN521 (Figure 28D).

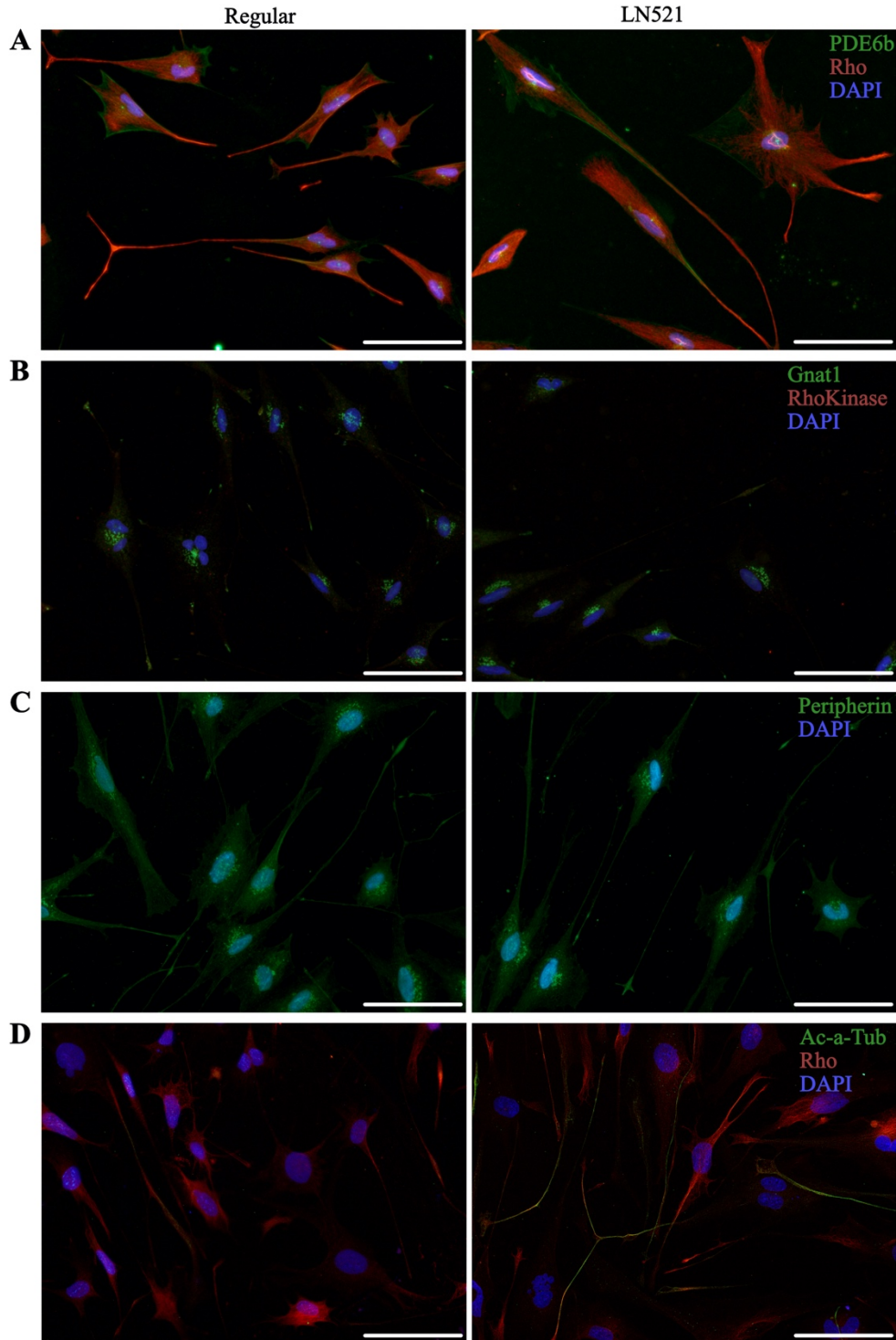


Figure 28. Immunofluorescence analysis of rod photoreceptor specific markers on 661W-A11 cells differentiated on regular laminin or laminin LN521. (A) Analysis of Rhodopsin (red) and PDE6 (green). (B) Analysis of Rhodopsin (Rho) kinase (red) and GNAT1 (green). (C) Analysis of Peripherin (green) protein (D) Analysis of Rhodopsin (Rho) (red) and acetylated-alpha-Tubulin (green) protein. Nuclei were stained with DAPI (blue). Scale bar: 100 μ m.

To sum up, we defined a protocol for differentiation of 661W-A11 cells that requires the use of DMEM-F12 medium, withdrawal of serum, addition of bFGF, retinoic acid, sodium butyrate, taurine and N2 and LN521 as substrate. The most evident differences

among cells differentiated on different types of laminins were the sizes of the cell bodies and the filaments that were much longer in cells differentiated on LN521.

4.3.2. Mimicking photoreceptor degeneration on differentiated 661W-A11 cells

Rods carrying the recessive mutation in the *PDE6B* gene have dysfunctional PDE6 enzyme that leads to an increased intracellular calcium level due to increased cGMP, resulting in activation of PKG and calpains. The activation of these pathways was demonstrated in 661W-A11 cells [371]. Here, we wanted to evaluate if differentiation could improve the cell model and could provide a better system to mimic photoreceptor degeneration *in vitro*. According to this purpose, a zaprinast-based treatment was performed to 661W-A11 cells after five days of differentiation (Figure 29A).

The previously developed zaprinast treatment protocol (see chapter 4.1.3) was applied to differentiated 661W-A11 cells at 300 μ M, 400 μ M, and 500 μ M for 24 and 48 hours. We examined the concentration-dependent cell viability at both exposition times by MTT assay. Differentiated 611W-A11 cells at the concentration of 400 μ M for 24 and 48 hours demonstrated similar cell viability reduction, around 30%, as found in 661W-A11 cells (Figure 29B). We selected the 400 μ M zaprinast concentration for further cell death assessments. Ethidium homedimer staining assay was used to determine cellular death in both zaprinast treated and not treated differentiated 661W-A11 cells. We found that 400 μ M zaprinast treatment for 24 hours caused almost 30% cell death in differentiated 661W-A11 cells (Figure 29C and 29D).

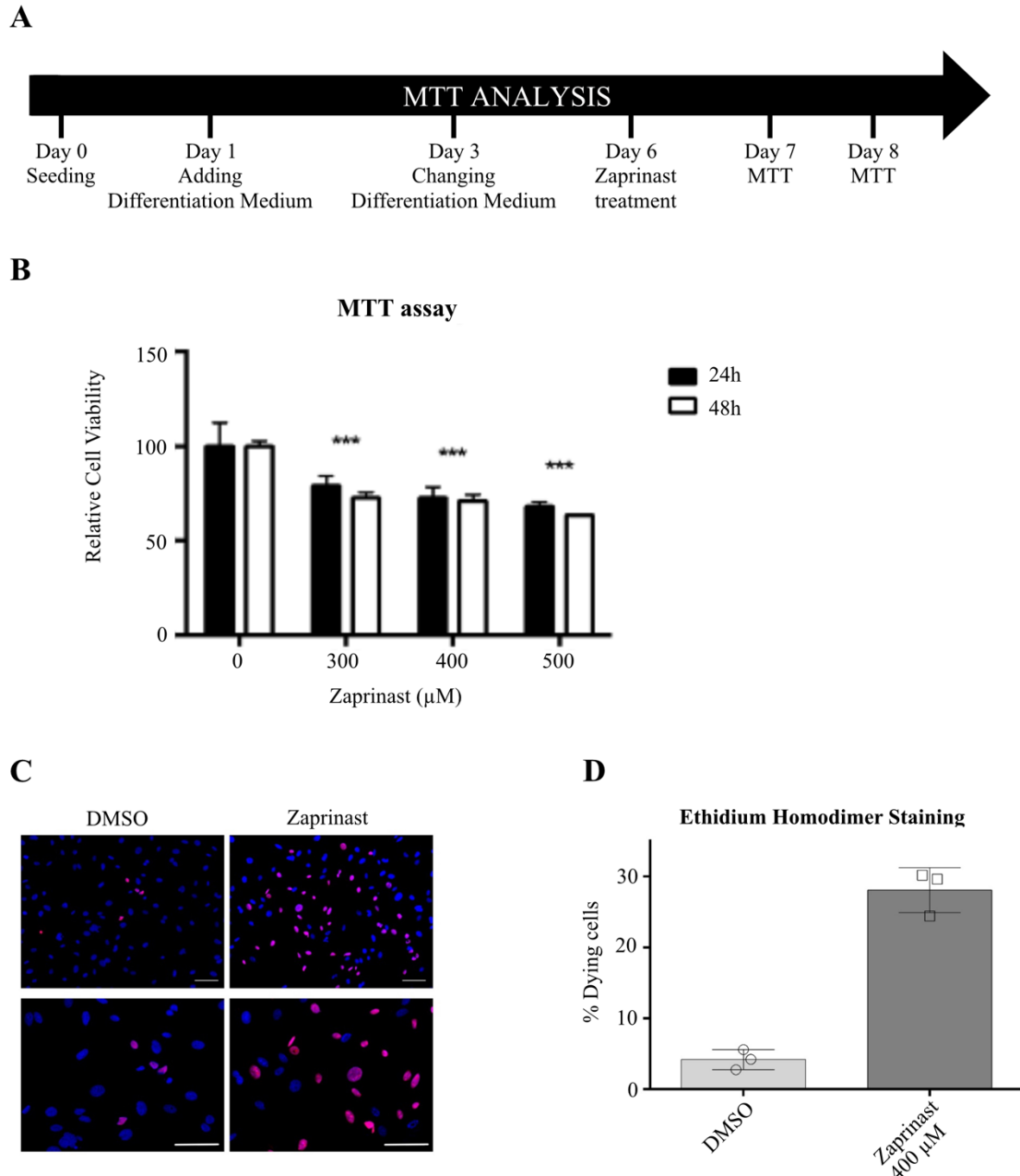


Figure 29. The treatment of 661W-A11 cells with the PDE6 inhibitor zaprinast after differentiation decreases cell viability and increases cell death. (A) Workflow of cells treatments (B) Cell viability, assessed by MTT analysis, is reported for 5 days differentiated 661W-A11 treated with zaprinast at 300 μM, 400 μM and 500 μM for 24h and 48h. (C) Fluorescence microscopy images and (D) quantification of ethidium homodimer positive 661W-A11 cells treated with zaprinast at 400 μM or DMSO (control). Ethidium homodimer staining analysis was performed after 24h zaprinast treatment on cells differentiated for 5 days. Scale bar: 100 μm; one-way ANOVA, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

We then wanted to analyze the cell death mechanism as in 661W-A11 cells after zaprinast treatment to define if we were mimicking PDE6 loss of function mutation.

We assessed PKG activation analyzing the PKG target VASP that is phosphorylated by PKGs at Serine 239. We performed immunofluorescence analysis labeling with an anti-

p239VASP and confirmed activation of PKG in the zaprinast treated differentiated cells (Figure 30A). Additionally, we counted p239VASP stained cells to quantify the data (Figure 30B). Our results showed that zaprinast treatment resulted in around 25% of cells activating PKG, a value in the range of the percentage of dying cells found by ethidium homodimer staining.

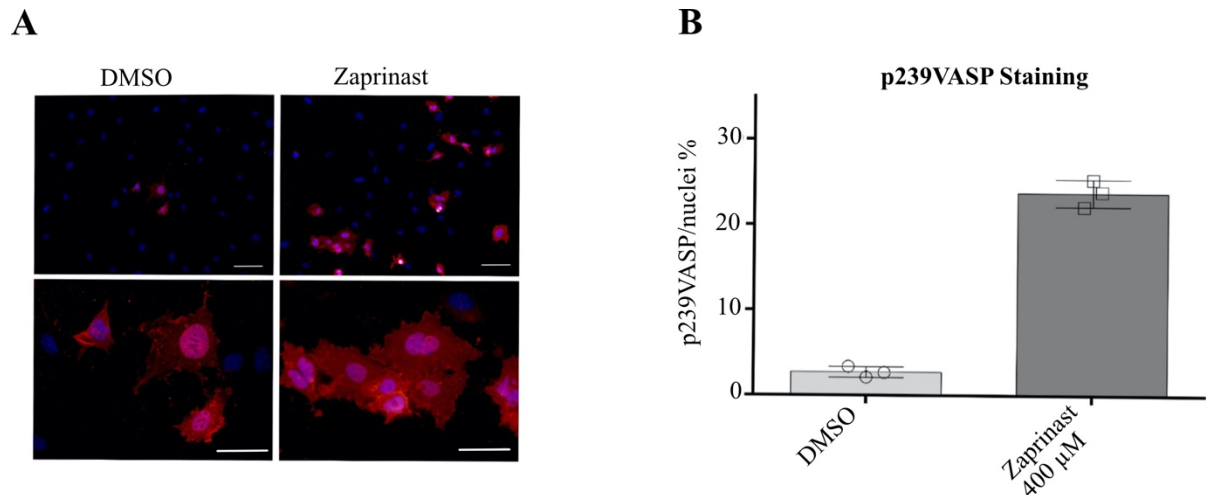


Figure 30. Immunofluorescence analysis of p239VASP on zaprinast treated 661W-A11. 661W-A11 cells were treated with zaprinast at 400 μ M concentration for 24h after 5 days of differentiation. (A) Fluorescence microscopy images of zaprinast and DMSO treated cells (control); p239VASP was stained in red and nuclei were stained in blue (DAPI). Scale bar: 100 μ m. (B) Quantification of p239VASP positive cells on DAPI positive nuclei.

Elevated intracellular calcium is also a common feature in RP mutant photoreceptors. Previous *in vivo* studies in animal models showed that increased calcium causes activation of calpain protease and this was also demonstrated in 661W-A11 cells [38–40]. We, thus, assessed calpain protease activity in five days differentiated 661W-A11 cells treated at 400 μ M zaprinast for 24 hours. We compared DMSO (control) and zaprinast treated differentiated 661W-A11 cells using the *in situ* calpain activity assay which is based on a fluorescent molecular probes (7-amino-4-chloromethylcoumarin, t-BOC-Leu-Met) that can be detected, when cleaved by calpains, at the fluorescence microscopy. We found an increase in cells with activated calpains (Figure 31A-B). Approximately 20% of zaprinast treated cells were labelled. Our data confirmed that differentiated 661W-A11 cells showed similar sensitivity to zaprinast treatment as 661W-A11 and can be used as an *in vitro* model for molecular studies on RP.

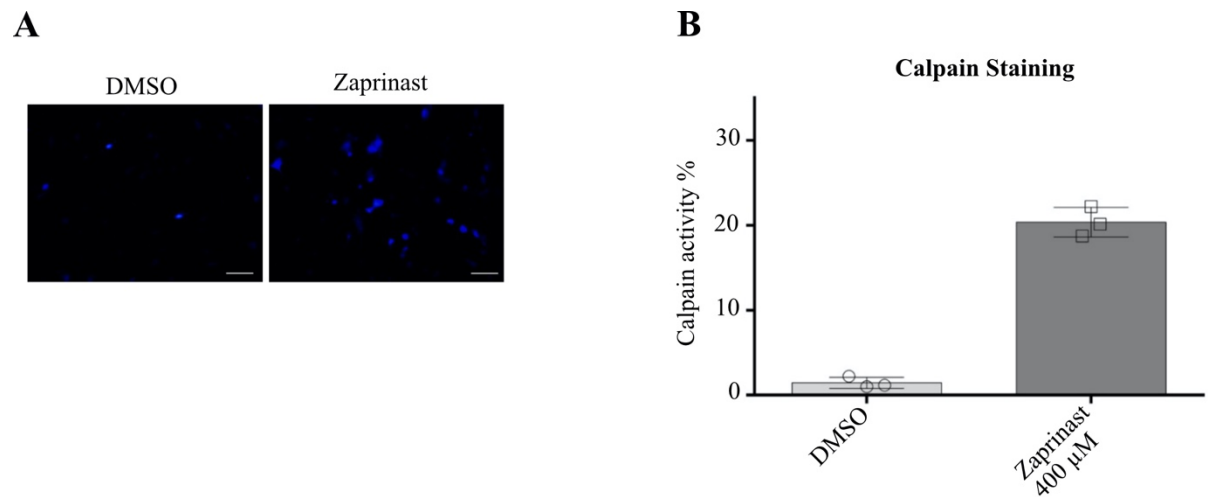


Figure 31. Calpain activity assay on zaprinast treated differentiated 661W-A11 cells. 661W-A11 cells were treated with zaprinast at 400 μ M for 24h after 5 days of differentiation. (A) Fluorescence microscopy images of calpain activity staining in zaprinast treated cells and DMSO treated cells (control). (B) Quantification of the number of cells showing calpain activity in zaprinast treated cells and DMSO treated cells (control). Scale bar: 100 μ m

5. DISCUSSION

An increasing number of studies have suggested that photoreceptor cell death is triggered by activation of cGMP-dependent intracellular targets in various IRD forms [8]. Several mutations associated with retinal dystrophies, such as RP40 and LCA4, have been linked to cGMP-dependent photoreceptor cell death [106, 373]. Notably, a high level of cGMP dysregulation may initiate downstream events, and these events end up with toxic outcomes in photoreceptor [33]. In this study, a cell line with rod-specific characteristics was generated and inhibition of PDE6, which increased cGMP and calcium levels, was able to trigger in this new cell line cell death pathways previously identified in animal models of RP [3]. We need to keep in mind that there are some limitations in this *in vitro* system but, while probably not all cell death pathways in this *in vitro* model were simulated, results showed that the 661W-A11 cell line could reproduce rod photoreceptor cell death connected to elevated intracellular cGMP. The limitation of the *in vitro* model presented here needs to be considered when using this system for high-throughput screening.

A link between calcium influx and photoreceptor cell death in retinal degeneration was previously demonstrated. High quantities of cGMP are thought to cause calcium influx through CNGC, and too much calcium causes photoreceptor cell death [374]. Two significant channels that play a role in calcium influx are detected in photoreceptors, namely the CNGC in the OS of the photoreceptor and the voltage-gated calcium channels in the body of photoreceptor cells [375]. L-type voltage-gated channel are targeted by benzothiazepine drug known as D-*cis* diltiazem. This drug was applied to 661W-A11 cells, and reduction of the zaprinast-induced calcium influx was observed [371]. The neuroprotective impact of D-*cis* diltiazem on RP models is contradictory. An earlier study revealed that D-*cis* diltiazem might prevent rod degeneration in the *rd1* mutant mouse. Although some investigations validated these findings, others could not replicate its protective effects [41, 70, 376]. In our model, D-*cis* diltiazem treatment decreased intracellular calcium, suggesting that calcium enters in these cells mainly through the L-type channel. Furthermore, D-*cis* diltiazem can also have a moderate effect on CNGC [377], so a possible calcium influx through CNGC cannot be excluded, as the study found the expression of mRNA encoding the CNGC subunits in the cells [371].

Activation of PKGs plays an important role in cell death in some neuronal cell types [77]. Many studies have shown that cGMP-dependent PKG overactivation can trigger cell

death [61]. In mammals, two different genes encode PKG: *PRKG1* for the isoforms PKG1 α and PKG1 β , and *PRKG2* for PKG2 [378]. Expression of PKGs has been found in the retina, and the activity of PKG has been studied in 661W cells [361, 379, 380]. The importance of PKG in the cell death process was established by PKG stimulation and in a CNGC loss-of-function mouse model in which *Prkg1* knock-out led to sustained rod cell survival [381]. Harmful effects to the cells exposed to PKG activation in response to an increase in intracellular cGMP have been also demonstrated. Interestingly, 661W-A11 cells produced poor tolerance to PKG2 activators [371], these are compounds previously characterized as PKG activators in cancer cell lines [82, 83, 382, 383].

To sum up, 661W-A11 cells may be utilized as a cell model to study the photoreceptor cell death mechanism based on cGMP-dependent PKG activation and for high-throughput drug screening. In the study presented in this thesis we used the new *in vitro* model for identification of gene expression changes related IRD caused by increased cGMP. The advantage to use the mRNA from a cell line instead of mRNA extracted from degenerating retinas is that, by the use of an acute stress, you can have more photoreceptors cells in the dying phase, and cells are better synchronized. In fact, the percentage of cells labelled by TUNEL at the peak of cell death in different IRD models is quite low [75].

The RNA-Seq data analysis indicated that the most affected genes were related to the cholesterol biosynthesis and metabolism pathways. A link between the unbalance of cholesterol homeostasis and AMD has been previously demonstrated [138], thus it may play a role also in IRD. Specifically, the downregulation of the *Srbef2* gene attracted our attention, because *Srbef2* is a member of membrane-binding transcription factors genes that encode the SREBPs. It is a master regulator of cholesterol and fatty acids metabolism [127]. Another key finding from RNA-Seq data was the downregulation of *Hmgcr* that encodes an enzyme at the early steps in the cholesterol biosynthesis pathway. The *Hmgcr* gene expression is regulated at the transcriptional level by the SREBP [148]. Importantly, downregulation of both these genes was also confirmed in the *rd10* mouse, the *in vivo* model that was better simulated by the zaprinast stress in 661W-A11. Otherwise, these two genes appeared to be upregulated in the *Rho*^{P23H/+} mutant degenerating retina. If indeed SREBF2 activates *Hmgcr*, the finding that both are upregulated is not surprising. SREBPs control intracellular cholesterol and fatty acids levels through a regulatory feedback system mediated by a family of transcription factors [384]. SREBPs are synthesized as inactive precursors bound to

membranes of the ER and require cleavage for translocation to the nucleus and binding to the promoter regulatory elements [385]. P23H mutation in rhodopsin accumulates and can affect the ER. There is, thus, also a post-transcriptional regulation of these transcription factors and further biochemical studies are required to better characterized SREBF2 function in photoreceptor degeneration.

MVK is an essential enzyme in the mevalonate pathway located downstream to HMGCR and is also regulated by *Srbef2*. We observed that *Mvk* is downregulated in 661W-A11 cells exposed to zaprinast. It was also slightly downregulated in *rd10* even if not yet significant. Due to the availability of mutant mice, we could assess changes in expression in only two biological replicates for *rd10* and *Rho*^{P23H/+}. The analysis of more mutant animals will allow us to define if expression of this gene was reduced also in the degenerating retina, as found in the cell model. Interestingly, *Fdps* is significantly downregulated in zaprinast treated 661W-A11 cells and in *rd10* but not in *Rho*^{P23H/+} retinas. On the other hand, *Nsdhl* was downregulated in the cell system and slightly downregulated in *rd10*, whereas significantly downregulated in *Rho*^{P23H/+} model. While singular mutations could affect cholesterol biosynthesis differently, further studies may be needed to find distinct mutations outcomes related to cholesterol biosynthesis.

High quantities of cholesterol accumulation in RPE cells are thought to cause photoreceptor cell death, depending on the dysfunction of the influx and efflux of cholesterol mechanisms through ABCA1/ABCG1 transporters. While expression of these two transport proteins are significantly higher in healthy RPE cells, ABCA4 protein expression is higher in healthy photoreceptor cells [237, 386, 387]. Interestingly, our data showed that expression of the *Abca2* gene was downregulated in zaprinast treated 661W-A11 cells, as well as in the *rd10* animal model. This gene is primarily expressed in CNS and macrophage cells and plays a role in intracellular trafficking and metabolism of LDL-derived cholesterol and sterol-related molecules [388] [237, 386, 387]. Downregulation of *Abca2* may be a significant finding for the mutant animal model. Any cholesterol accumulation in the photoreceptor cells could directly affect the phototransduction cascade. Previous studies showed that cholesterol accumulation in the outer segment and cholesterol distribution changes in disc and plasma membranes can affect rhodopsin activation. Future experiments should be focused on the confirmation of ABCA2 changes in the degenerating retina.

When we checked the effect of CN03 treatment on the expression of cholesterol related genes, we found a slight increase even if not significant. These promising preliminary results solicit deeper studies on the cholesterol pathway during photoreceptor degeneration.

The most downregulated gene in the RNA-Seq analysis was *Postn* (Periostin). Periostin is an extracellular matrix protein and it serves several roles in health and disease including eye diseases like diabetic retinopathy, AMD and glaucoma [389]. Among other functions, Periostin mediates response to tissue damage [390, 391] and, interestingly, plays a role in lipid metabolism in kidney, where cholesterol was found at lower levels in *Postn* knock-out mice [392].

We identified some other genes not related to the cholesterol pathway that were significantly changed in the RNA-Seq analysis.

One of these genes is *Axin2* that was found downregulated in 661W-A11 cells after zaprinast treatment. *Axin2* gene expression is an indicator of the canonical Wnt signalling pathway activation [393]. Wnt signaling is essential in the regulation of diverse biological processes such as development, apoptosis, nervous system function and tumorigenesis [394]. We validated the data of RNA-Seq on *rd10* where *Axin2* was downregulated indicating a dysregulation in the Wnt signaling pathway leading to retinal degeneration in *rd10*. This preliminary result is not in line with previous studies showing that the Wnt signalling pathway is activated during photoreceptor degeneration in *rd1* mice [395]. Oppositely, another study suggested that there is a correlation between dysregulation of the Wnt signaling pathway and neurodegeneration [396]. Moreover, our RNA-Seq analysis pointed out that other genes related to Wnt pathways, such as *Wnt5a*, *Wnt9a*, *Wnt7b*, *Lef1*, *Fzd2* or *Dkk3* were decreased. Further studies should evaluate the Wnt pathways in degenerating retinas to better understand its role in association to the cGMP pathway. Interestingly, a partial recovery of expression upon CN03 treatment in 661W-A11 cells was detected only for *Wnt7b*.

Finally, *Hk2* (Hexokinese 2) was differentially expressed in 661W-A11 cells exposed to zaprinast. Interestingly, this gene appears to be downregulated in both *rd10* and *Rho*^{P23H/+} models. HK2 is one of the main regulators of aerobic glycolysis. Moreover, it is involved in the regulation of apoptotic signaling, and it was shown to be required for survival during aging and outer retinal stress. Previous studies observed that knock-down of *Hk2* in rod

photoreceptors leads to age-related photoreceptor degeneration. Therefore, HK2 appears to be important for preserving photoreceptors during acute nutrient stress and aging [397, 398]. Our data cannot address the question on the exact role of HK2 in photoreceptor physiology and, unfortunately, we did not detect recovery of *Hk2* expression in CN03 treated samples. Further studies need to be performed on the retinas from the murine models to confirm these preliminary results.

The failure to retrieve transcriptional changes upon CN03 treatment based on adjusted p-value, can be explained by the fact that, in this experiments, we exposed the cells only for a short time to CN03. Therefore, CN03 may have a rescue effect on the cell system at the level of gene expression but we could not detect it so far. We expect that it might become evident after a longer time of exposure of the stressed cells to the compound.

Another point that should be discussed is the fact that we were unable to confirm changes in expression of genes related to cholesterol in the *rdl* mouse model. *rdl* is characterized by a fast retinal degeneration. We collected RNA at PN11, which is the peak of photoreceptor degeneration for this model [75]. At PN11 the rod outer segment has not developed yet and being cholesterol particularly important for the outer segment it is possible that these enzymes are not majorly involved in the photoreceptor metabolism. We should perform a transcript quantification for the cholesterol related enzymes at different postnatal times in the murine retina to assess this hypothesis.

In this thesis, we developed a differentiation protocol to improve the efficiency of the 661W-A11 cell model to generate a system able to better mimic rod degeneration, as presented at the beginning of the study. Our data showed that DM4 was the most valuable medium because provided higher differentiation efficiency in five days (about 70%) out of four media tested on 661W-A11 cells. Additionally, we observed that the LN521 substrate provided a stable differentiation condition when we compared it with the other substrates. The culture of 661W-A11 cells on LN521 for five days resulted in most cells acquiring a neuronal phenotype and elongated filaments with localization of rod-specific proteins. Our data confirmed a previous report that used LN521 to differentiate iPS cells into rod-like cells [399]. While 2D *in vitro* differentiation could be easily influenced by culture conditions, we used this downside to positively impact differentiation with LN521 coated plates. In fact, we need to point out that, in previous studies, culture conditions were highly heterogeneous and allowed lower differentiation efficiency compared to the protocol described here.

Furthermore, we mimicked a PDE6 loss function mutation on the differentiated 661W-A11 cells by stress with zaprinast to be compared to the published results on 661W-A11 cells without differentiation [371]. We found that differentiated 661W-A11 cells showed the same sensitivity to zaprinast treatment at 400mM zaprinast concentration for 24 hours. These data indicated that differentiated cells treated with zaprinast may also model photoreceptor degeneration characterized by elevated levels of cGMP and calcium, causing PKGs and calpains activation [44, 61, 400]. Based on immunofluorescence analysis, we confirmed activation of PKGs and calpains on differentiated 661W-A11 cells. According to all these findings, mimicking rod photoreceptor degeneration was achieved on differentiated 661W-A11 cells that represent a closer model to rod photoreceptors in the retina.

The lack of *in vitro* models simulating rod photoreceptor degeneration hampered the development of new therapeutic approaches and high-throughput screens of drugs as well as research on cell death mechanisms. Several animal models and cell lines are available; however, they are not always appropriate for systematic studies. Some *in vitro* systems take a long time to differentiate into rod-like cells, such as ESC or iPSC and are thus characterized by high heterogeneity. Generation of rod photoreceptors *in vitro* from a cell line can open new approaches for the study of IRDs.

6. CONCLUSIONS

This chapter concludes the thesis study by summarizing primary research outcomes, relationship to the research aims, the value of the study, and contributions. It will also review the limitations of the study and purpose opportunities for future studies.

RP is the most common form of the IRDs, causing incurable blindness. More than 100 genes have been linked to photoreceptor degeneration. The high heterogenetic background of RP hampers the development of treatments for any possible cure. At the molecular level, a more profound elucidation of the mechanisms underlying the progression of the disease is needed for the development of new treatments for patients.

The main focus of the thesis study was the characterization of gene expression changes during rod photoreceptor degeneration to shed light onto rod photoreceptor cell death mechanisms.

For this purpose, we developed a new *in vitro* model using the 661W cell line and validated a stress protocol based on zaprinast to model the PDE6B gene loss of function mutation as in RP patients and related animal models.

In this system, we looked for differentially expressed genes by comparing the cells exposed to zaprinast versus not treated cells, giving us a screenshot of transcriptional changes during rod-like cell degeneration. The RNA-Seq analysis highlighted significant downregulation of genes mainly related to cholesterol biosynthesis and metabolism. Cholesterol is a fundamental lipid component of the membranes of the rod outer segment and, additionally, for rhodopsin functionality. Further studies are necessary to identify the relationship between the mutation causing degeneration and the genes linked to cholesterol metabolism.

Finally, we developed a differentiation protocol to improve the 661W-A11 *in vitro* model. Generating mature rod-like cells could provide an advanced model for drug screening. We increased the expression of rod-specific proteins and the elongated cell shape phenotype and demonstrated that upon the PDE6 inhibition the newly generated cellular system can appropriately simulate the disease.

This study filled the gap in the field of IRD that is the lack of *in vitro* models representing rod degeneration. We should consider, as a limitation, that this cell system only

represents the PDE6 loss of function that causes increased cGMP and calcium levels. The gene characterized expression changes highlighted new molecular events that require further studies to be validated in the degenerating retina of animal models and more profound molecular studies to find their role in rod photoreceptor degeneration.

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