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Genes and proteins controlled by cGMP-PKG during retinal degeneration

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Genes and proteins controlled by cGMP-PKG during retinal degeneration

Jiaming Zhou



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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended at on 7 of December at 09.00 in Ögonklinik A, Kioskgatan 1, SUS Lund

Faculty opponent

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The Paper I showed the cGMP-PKG-dependent transcriptome in this study. Applying RNA sequencing to study the retinal explants from the diseased *rd1* models and WT with cGMP-PKG manipulation, I identified the cGMP-PKG-dependent genes and proposed that this system may negatively regulate oxidative phosphorylation and mitochondrial pathways, which may affect retinal degeneration.

The paper II investigated the cGMP-PKG phosphoproteome. The phosphorylated peptide enrichment and mass-spectrometry were applied to explore the cGMP-PKG-dependent phosphoproteome within *rd1* retinal explants with PKG inhibition or not. I identified a list of cGMP-PKG-dominated phosphorylations and picked up RAF1 proto-oncogene, serine/threonine kinase (RAF1) for further validation. This suggested that RAF1 may be involved in retinal degeneration, although in an as yet unclear mechanism.

The Paper III investigated cGMP-PKG-dependent kinase activity profiling and the phosphoproteome with a microarray-based technique and mass-spectrometry, respectively. The *rd10* model, with a different mutation in the gene for PDE6 was used. This yielded the lists of cGMP-PKG-dependent kinase and phosphorylations, which were partially compatible with Paper II. Also, this showed that Ca²⁺/calmodulin-dependent protein kinase II and IV (CaMK2, CaMK4) may play a role during retinal degeneration.

Paper IV focused on cyclin-dependent kinase 1 (CDK1), which was identified from Paper II, namely, and investigated if it has effects on retinal degeneration. The data showed that CDK1 participates in the late stage of retinal degeneration, and also provided a link between this enzyme and the cGMP-PKG system.

The Paper V validated another target, pyruvate kinase isozyme M2 (PKM2) identified in the previous transcriptome study. The PKM2 within retinas was activated from two disease models, namely *rd2* and *rd10* in a pharmacological manner during explant culture. I observed that PKM2 activation in *rd10* alleviated the photoreceptor degeneration while no difference was noticed in *rd2* under treatment.

All in all, this thesis provides novel insights about cGMP-PKG-dependent targets, which may have a role during photoreceptor degeneration and cast light on the therapeutic development of this retinal disease.

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To the people fighting against Retinitis Pigmentosa

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Abstract

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List of Papers

Paper I

Zhou, J., Rasmussen, M. and Ekström, P. (2021) “cGMP-PKG dependent transcriptome in normal and degenerating retinas: Novel insights into the retinitis pigmentosa pathology,” *Experimental Eye Research*, 212, p. 108752.

Paper II (manuscript submitted for publication)

Zhou, J., Welinder, C and Ekström, P. The phosphoproteome of the degenerating *rd1* retina changes after protein kinase G inhibition.

Paper III (manuscript)

Roy, A*, Zhou J*, Nolet M., Welinder C., Zhu Y., Paquet-Durand F., Groten J., Tomar T and Ekström P. Integrative kinase activity profiling and phosphoproteomics of retinal explants during cGMP dependent retinal degeneration.

*These authors contributed equally

Paper IV

Zhou, J. and Ekström, P. (2022) “A potential role of Cyclic Dependent Kinase 1 (CDK1) in late stage of retinal degeneration,” *Cells*, 11(14), p. 2143.

Paper V (manuscript submitted for publication)

Zhou J and Ekström, P. Pyruvate kinase 2, an energy metabolism related enzyme, may have a neuroprotective function in retinal degeneration.

Introduction

The eye is the organ to send visual information, such as color and image, to the brain. Vision loss caused by any reason would thus severely affect an individual's quality of life, and also lead to a national or even global burden¹. This thesis focuses on one group of eye diseases, Retinitis Pigmentosa (RP), and an introduction is given with the aim of providing a brief description of retinal structure and biology, phototransduction, RP, and the players cGMP-PKG, CDK1, and PKM2.

Retinal structure and biology

The eye anatomy can be summarized as three layers and three chambers². The distinct layers include the outer layer, formed by the cornea and sclera, the middle layer, comprised of the iris and pupil, and the inner layer, which consists of the retina. The space between the cornea and iris is termed the anterior chamber, while the posterior and vitreous chamber represents the room between the iris and the lens, and the one between lens and retina, containing the vitreous humour or vitreous, respectively² (Figure 1).

The retina is a layered structure with diverse cell types with specific morphology and function³. The development of imaging techniques and immunostaining-based labeling have deepened our comprehension of the retinal structure and its integrated cell components, respectively⁴. A schematic section (Figure 1), reveals that the retina consists of three layers of cell bodies and two layers of synapses^{3,5}. Photoreceptors, including rods and cones, are the predominant cells lying in the outermost part of retinas, whose cell bodies form the outer nuclear layer (ONL)⁶. The outer segments of the photoreceptors are enveloped by the so-called retinal pigment epithelial cells (RPE), which form a simple layer of cuboidal cells with essential physiological functions, such as active participation in photoreceptor membrane turnover, nutrient transportation to photoreceptors, and involvement in the visual cycle⁷. The cell body layer in the intermediate position is the inner nuclear layer (INL) with three main identified cell categories consisting of bipolar cells, horizontal cells, and amacrine cells^{5,8}. The ganglion cells, functioning as the main output neurons, lie in the innermost retina and form the ganglion cell layer (GCL), and their axons in turn form the nerve fiber layer, which is the layer closest to the vitreous⁹. The outer plexiform layer (OPL) points to the network of synapses

between ONL, specifically rod cell endings and cone cell branched foot plates, and various bipolar cells or horizontal cells in INL¹⁰. Another synaptic layer, namely the inner plexiform layer (IPL), delivers signals from varied cells within INL to ganglion cells¹¹.

The light perception is processed via two main identified pathways. The pathway originating from the rods and their connected cells through the retina is responsible for night vision perception^{12,13}. In the dark, the rods are depolarized and release a high basal amount of glutamate, which will have inhibitory effects on bipolar cells. When light strikes the pigment of rods, the rods turn hyperpolarized so that the release of glutamate is reduced¹⁴. Then the bipolar cells become depolarized¹⁴. The horizontal cells play a role in the modulation of this synapse¹⁵. Then the so-called amacrine cells receive the signal input from rod bipolar processes on their arboreal dendrites¹⁶. The terminal parts in this pathway are the ganglion cells.

The color vision perception is conducted by pathways in the cone system¹³. As the sensors of different wavelengths of light, the cones convey the bright light signal via releasing glutamate to cone bipolar cells and subsequently to ganglion cells¹⁷. Then the ganglion cells receive these chemical signals, specifically at the transmembrane receptors, and transform them into electrical signals¹⁸, that are delivered through the optic nerve to the brain visual centers¹⁸.

Structure of the Retina

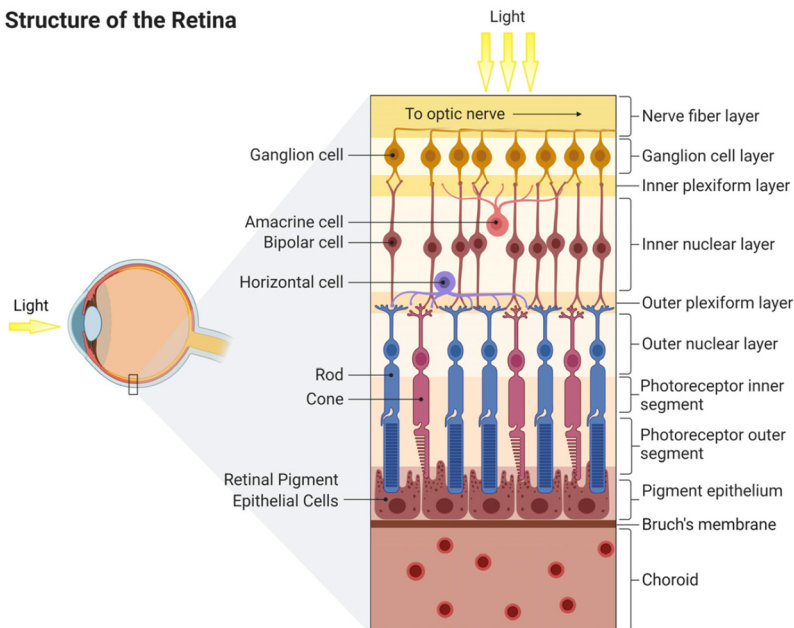


Figure 1:

Structure of the eye and retina, with the schematic sections of highlighted layers and cell types. The figure is reprinted from a published thesis by Rasmussen 2022.

Phototransduction

The vision perception starts with a biological complex, that performs the phototransduction, which occurs in the outer segment, a dynamic structure with enriched stacks of membranes. Multiple enzymes with a series of biological alterations are involved in this cascade. Rhodopsin, a seven transmembrane domain protein belonging to the G-protein-coupled receptor family is regarded as the primary protein to initiate phototransduction¹⁹. Structurally, rhodopsin consists of two main parts, the protein part opsin with the function property of conveying the light detection, which is carried out by the other part, the attached chromophore 11-cis-retinal. In the dark, the 11-cis-retinal is bound to opsin, functioning as an antagonist and holding it in an inactive state¹⁹. When the photon reaches the outer segment and is absorbed by rhodopsin, the 11-cis-retinal is isomerized into the all-trans configuration to induce the structural changes of opsin and activate it¹⁹. As a G-protein-coupled receptor, the activated opsin binds and activates the peripheral membrane heterotrimeric G-protein transducin²⁰. A significant signal amplification happens in this step, as approximately 400-800 transducins would be activated by single opsin²¹.

The next effector of the cascade is cGMP-phosphodiesterase (PDE), whose structure is made of heterodimeric catalytic subunits (α and β) and the inhibitory γ subunits. Transducin transmits the signal and stimulates the activation of PDE via inducing the dissociation of the inhibitory γ subunits from the PDE complex²². The role of PDE is to hydrolyze the cyclic guanosine monophosphate (cGMP) and produce guanosine monophosphate (GMP), resulting in decreased intracellular cGMP levels. This is achieved via the high affinity of activated PDE isoforms to bind and/or dimerize cGMP²². It is well documented that high levels of photoreceptor cGMP bind to cGMP-gated cation channels in the dark, and subsequently the channels are maintained in an open state to allow Na^+ and Ca^{2+} inflowing to the segment²³. Thus the intracellular Na^+ is at a relatively high level to keep the photoreceptor depolarized²³. Therefore, in the photoactivation situation, where a drop in cGMP occurs due to high PDE catalytic activities, the cGMP-gated cation channels become “closed”²³. As the ion influx ceases because of the channel closure, a decrease in intracellular Na^+ occurs, which leads to membrane hyperpolarization of photoreceptors, and decreases the glutamate release at the synaptic terminal²³. The signal is further transmitted among different retinal neurons as mentioned in the above section.

Following the light activation, the photoreceptor will be in a recovery stage, where the activated components, such as rhodopsin, transducin, and PDE turn into inactivation at this period. It is worth mentioning that Ca^{2+} -mediated mechanisms play an essential role to regulate and balance these protein levels. For rhodopsin, two proteins, namely rhodopsin kinase (GRK1) and arrestin directly contribute to

the inactivation. GRK1 phosphorylates the rhodopsin and lowers its activities, and this reaction is regulated by recoverin, a type of calcium-binding protein²⁴. Specifically, in darkness when the Ca^{2+} levels are high, the Ca^{2+} binds to recoverin and GRK1 to form a complex to initiate the rhodopsin phosphorylation, which decreases the rhodopsin activities²⁵. Then the arrestin further blocks the catalytic activities of rhodopsin via binding the phosphorylated form of this protein²⁶. The inactivation of transducin is achieved via intrinsic GTPase activities, like most heterotrimeric G proteins. To be more specific, a GTPase-activating protein (GAP) complex, which consists of RGS9 (regulator of G-protein-signaling isoform 9) and G-protein β -subunit or G-protein β -subunit-like protein, is responsible for hydrolyzing the GTP of the transducin α -subunit, leading to the inactivated state of transducin^{27,28}. This further facilitates the inactivation of rod-specific cGMP-phosphodiesterase 6 (PDE6), while a GAP-independent mechanism to inactivate PDE6 via the effects of the PDE6 γ subunit was also reported^{29,29}. The cGMP, therefore returns to normal, high levels when the action of PDE6 is decreased²². Another contributor to the restoration of the cGMP within photoreceptors adapting to darkness from light is the guanylate cyclase (GC), with the function to generate cGMP from triphosphate (GTP)³⁰. The Ca^{2+} is again an important regulator of GC, as it is regarded to have inhibitory effects on GC³⁰. This is supported by the fact that low intracellular Ca^{2+} will stimulate GC activation, via the interaction with guanylyl cyclase-activating proteins (GCAP)^{29,30}.

All in all, this phototransduction in the light-dark circle is an essentially biological process for visual perception, and a disorder of the components of this cascade may generate vision loss, including via retinal degeneration.

Retinitis Pigmentosa

Retinitis Pigmentosa (RP) refers to a group of inherited retinal disorders, with the symptom of progressive vision loss³¹. Typically, the clinical presentation starts with peripheral vision issues and reduced night vision, followed by decreased visual fields, and in some cases, total blindness by the end³¹. Although the mutations of disease-leading genes vary, as over 70 RP-related genes were documented (<https://sph.uth.edu/retnet>, latest updated on 9 of June 2022), the dysfunction and loss of photoreceptors are regarded as the main reason for visual impairment^{32,33}. At present, a reported prevalence of over 1:3000³², particularly in industrial countries, makes it one of the leading causes of vision loss. As a rare disease, RP is gaining widespread public concern, since there is in principle no effective cure, with the only exception of the recently approved RP65 gene therapy³⁴.

According to the inheritance characteristics, around 25% to 30% of cases of RP belong to autosomal dominant retinitis pigmentosa (adRP), which usually presents

the mildest form starting after the age of 50³⁵. To date, more than 1000 mutations in 25 genes are reported within this category³⁵. The mutations in the gene for rhodopsin are most commonly seen, with over 100 mutations reported, which accounts for approximately 20% of adPRP cases³⁶. As the primary protein to initiate phototransduction, mutations in rhodopsin severely affect this biological process¹⁸. With the aid of molecular technique development, research has gained more insights into details of the mechanism of how rhodopsin mutant is detrimental to light perception. The indicated unfavorable affections include dysfunction of protein folding, complex formation with 11-cis retinal chromophore, G-protein activation of the rhodopsin protein, which may lead to neuron loss³⁷. It is worth mentioning that rhodopsin mutants are also associated with autosomal recessive RP (see below) at a lower frequency³⁶. Mutations in the gene pathogenic variants in pre-messenger RNA (pre-mRNA) splicing factor 31, PRPF31 are regarded as the second most commonly seen forms of adRP³⁸. Located in chromosome 19q13.42, the main function of PRPF31 is RNA processing, though the details regarding the molecular mechanism of PRPF31 mutants-leading RP are still unclear³⁸. Other known mutants belonging to adRP include peripherin2 (Prph2), RP1 protein (RP1) and more^{39,40}.

For the autosomal recessive (arRP) pattern of inheritance, which takes up 15-20% of the cases, RP symptoms typically start in the first decade of life⁴¹. Mutations of over 30 causative genes have been reported in this pattern, with the most well-understood one of retinal pigment epithelium 65 (RPE65) which participates in the visual pigment regeneration^{41,42}. Acting as an isomerase in the RPE cells, the RPE65 functions via reconvertng all-transretinol to the 11-cis form during phototransduction and regulating the balance of varied forms of retinol⁴³. Therefore, mutations in RPE65 cause a decline in levels of 11-cis-retinal and subsequent dysfunction in RPE and photoreceptors⁴³. The mutations in another group of genes that encode the PDE6 complex are also the common form of arRP⁴⁴. As a regulator to hydrolyze the cGMP level during phototransduction, PDE6 consists of α , β and two γ subunits²², whose mutations will cause RP. Though the details are elusive, the high level of cGMP due to PDE6 mutations may cause rod deaths via disordered Ca^{2+} influx into rods, while other mechanisms, such as abnormal high activity of cGMP-dependent protein kinase G, are also proposed^{45,46}. The latter possibility is further explored in the present thesis. More genes, such as ATP-binding cassette subfamily A (ABC1), member 4 (ABCA4), with a function in retinal metabolism, and interphotoreceptor matrix proteoglycan 2 (IMPG2) that acts as the component of the retinal intercellular matrix, and more have been reported to cause arRP when mutated, though the prevalence is relatively lower^{47,48}.

RP can also be inherited in an X-Linked form (XLRP), which is seen in around 10-15% of patients⁴¹. Among the six reported gene loci, the Retinitis Pigmentosa GTPase regulator (RPGR) has been more extensively studied than others, and it encodes a GTPase-binding protein which is located in the outer segment and associated with a number of cilia-centrosomal proteins⁴⁹. The other genes include

Retinitis Pigmentosa 2 (RP2), RP6, RP23, RP24, and RP34, and the pathophysiology of most of these gene products are unclear except RP2, where insights into the role of its gene product suggests it to affect cone photoreceptor sensory cilium elongation⁵⁰.

RP also co-occurs with other diseases that affect other organs and tissues, which has been described as syndromic RP⁵¹. This is in contrast with the non-syndromic forms of RP, including adRP, arRP, and XLRP mentioned above, presenting only vision loss³¹. Usher syndrome (USH) is the most frequently seen syndromic RP, with symptoms of hearing loss and sometimes vestibular dysfunction also observed clinically at the early stage of this syndrome and RP later on⁵². To date, ten USH-related genes have been identified, whose mutations are responsible for one of the three USH subtypes^{52,53}. Another known syndromic RP, namely Bardet-Biedl syndrome (BBS), affects photoreceptors and multiple systems, and in most cases, individuals present obesity, postaxial polydactyly, cognitive impairment, hypogonadotropic hypogonadism, and renal malformations⁵⁴. Currently 20 causative genes have been reported, while BBS1 and BBS10 account for most cases, approximately 50%⁵⁵.

Given the genetically heterogeneous profile of RP and that mutations in the specific individual genes cause vision loss via varied pathways and mechanisms, it is challenging to develop an effective standard therapy for this disease³³. Currently, a variety of treatment strategies are in different stages of development or clinical trials, with an estimation of 131 drugs⁵⁶. Some candidates are highlighted as cell therapies, gene therapies, gene-modified cell therapies, and antibody therapies⁵⁶. However, except for the recently approved RPE65 gene therapy³⁴, these strategies have so far not been successful and RP patients can only receive supportive care without further effective treatment.

cGMP-PKG

Cyclic guanosine monophosphate, cGMP, is well-recognized as a unique second messenger molecule, whose signaling has been extensively studied in various tissues⁵⁷. Two known upstream pathways are related to cGMP formation, namely, nitric oxide (NO) and natriuretic peptides (NPs), which initiate the cGMP synthesis via activating soluble guanylate cyclase (sGC) and particulate GC (pGC), respectively⁵⁷. As was mentioned previously, one of the downstream effectors is CNG, with the function to regulate ion influx²³. Except for this, another effector, cGMP-dependent protein kinase G (PKG), whose role was first identified in cardiovascular physiology⁵⁸, has gained increasing attention. As a serine/threonine-specific protein kinase, the function of PKG is achieved via the phosphorylation of a series of downstream targets⁵⁸. At present, two main expression forms of PKG

were identified as PKGI and PKGII, while PKGI was further subcategorized as PKGI α and PKGI β according to their splice variants⁶⁰. Structurally, PKG consists of two subunits, each of which includes an N-terminal regulatory domain, an autoinhibitory sequence, two tandem cGMP binding sites, and a C-terminal catalytic domain⁶⁰. When PKGI is bonded to cGMP, a conformational change happens such that the autoinhibitory sequence is released, followed by the activation of this kinase⁶⁰. When activated, PKGI participates in vascular biology to regulate smooth muscle via decreasing Ca²⁺ release from intracellular stores, and its role in apoptosis was also reported^{60,61}. For PKGII, while more details of the function are to be elucidated, it is revealed that PKGII acts as an activator of lung epithelial Na⁺-channels to regulate salt transport⁶².

The abnormally high cGMP levels were reported in not only *rd1*, but also several other retinal degeneration models⁶³, and this provides the link between retinal degeneration and high cGMP. The cGMP-dependent CNG activation appears to be the prime effector for photoreceptor death⁶⁴. Increasing evidence indicates that cGMP-PKG may act as a prominent disease driver in photoreceptor degeneration. PKG, specifically PKGI is expressed in photoreceptors, according to in situ hybridization studies of the brains and retinas of mice⁶⁵, while the PKGII expression in photoreceptors is unclear. This provides the possibility of PKG functioning in photoreceptors. More convincingly, elevated PKG activities in degenerating photoreceptors from the CNG deficiency mice were observed and reported by Ma and his colleagues⁶⁶. In the same study, they treated the photoreceptors with PKG inhibitor or by experimental deletion of guanylate cyclase-1 (GC1) and found the alleviation of cell death via suppression of cGMP-PKG signaling⁶⁶. In another study, the systemic administration of liposome-formulated cGMP analogue, a type of chemically modified cGMP compound with high membrane permeability to inhibit PKG isoforms, exerted apparently neuroprotective effects in degenerating photoreceptors⁶⁷.

Taken together, the cGMP-PKG system plays a vital role during retinal degeneration, which makes it a valuable molecular target for novel biomarkers or therapy development. However, more revealing insights are required to answer one relevant question: Are there any cGMP-PKG downstream pathways that have direct detrimental effects on photoreceptor health? As has been reported, cGMP-PKG regulates multiple genes in diverse tissues and also thousands of phosphorylated proteins^{68,69,70}. Further understanding regarding whether these or other cGMP-PKG downstream targets participate in retinal degeneration requires more investigation.

CDK1 and PKM2

According to the high throughput studies in the thesis (Paper I and II presented below), a series of cGMP-PKG-dependent downstream targets that may be potentially related to photoreceptor cell death was generated. Among these targets, cyclin dependent kinase 1 (CDK1) and pyruvate kinase 2 (PKM2) were selected for further investigation regarding if they were involved in retinal degeneration.

CDK1 is known as the main member of the cyclin-dependent kinase family to drive the cell cycle in eukaryotic cell. Upon activation via the formation of a cyclin B-CDK1 complex, CDK1 functions to phosphorylate substrates related to the cell cycle⁷¹. Apart from its regulation of mitosis, CDK1 was documented with multiple functions, including transcriptional regulation, cell polarization, and DNA damage repair, as well as an involvement in cell death⁷². Though it is regarded that CDK1 is expressed in mitotic cells, interestingly the re-expression of this cell cycle-related kinase has been reported in post-mitotic neurons, which are known as non-dividing and terminally differentiated cells during degeneration⁷³. Hence, it is likely that CDK1 participates in cell death with an unknown mechanism that is independent of the cell cycle. Despite investigations showing that CDK1 causes neuron death in some neurodegenerative models, its role in RP, which is regarded as a type of neurodegenerative disease, is still unclear.

Pyruvate kinase is one of the key enzymes in glycolysis, where glucose is broken down to generate energy⁷⁴. This enzyme has four subtypes, including L, R, M1 and M2, which are encoded by two groups of genes⁷⁵. The M2 subtype of pyruvate kinase (PKM2) has been shown to be expressed within photoreceptors. Given the characteristics of a high metabolic rate in photoreceptors, the PKM2⁷⁶, with the function to route glucose metabolism to pyruvate and then having it enter the tricarboxylic acid cycle for energy production, is expected to exert vital effects in photoreceptors. In addition to its role in glucose metabolism, PKM2 was also reported with a variety of biological functions, including nuclear localization, regulation of gene expression and protein activities, and more^{77,78}.

In this thesis, whether or not there is an involvement of these two proteins in photoreceptor cell death was investigated with a special focus on their links to the cGMP-PKG system.

Aims

As the development of novel therapeutic strategies for RP is required, this thesis aims to gain more insights into potential molecular targets for drug development, with a special focus on cGMP-PKG downstream signaling at genetic and proteomic levels.

Paper I:

To explore the cGMP-PKG-dependent transcriptome via RNA sequencing using retinal explants from either mouse retinal degeneration models or corresponding wild type with inhibition and activation of cGMP-PKG, respectively.

Paper II:

To study the cGMP-PKG-dependent phosphoproteome via the application of combined phosphorylated peptide enrichment and mass spectrometry (MS), using retinal explants from a disease model with inhibition of cGMP-PKG.

Paper III:

To study the kinase activity profiling and phosphoproteomics of retinal explants during cGMP-dependent retinal degeneration.

Paper IV:

To elaborate on Paper II's findings and gain more insights into whether CDK1 is involved in retinal degeneration as a downstream target of cGMP-PKG.

Paper V:

To elaborate on Paper I's findings and study whether PKM2 has neuroprotective effects on photoreceptor cell death.

Methods

The pharmacological manipulation of the cGMP-PKG system, as well as of PKM2, via adding compounds to organotypic retinal explant cultures was performed by Jiaming Zhou at Ophthalmology laboratory, Department of Clinical Sciences Lund, Faculty of Medicine, Lund University, Lund, Sweden. These explants with different treatments were used to run RNA sequencing, phosphoproteomics via peptide enrichment plus MS, and kinomics via microarray-based measurement. The RNA sequencing was done with the assistance of the Center for Translational Genomics, Lund University. The phosphoproteomic and kinomic studies were performed by Charlotte Welinder at the Translational Proteomics at Medical Faculty, Lund University, and PamGene International B.V., respectively. The bioinformatics of the phosphoproteomic analysis, as well as the target validation via qPCR, immunostaining, and proximity ligation assay, with associated data analysis, were also conducted by Jiaming Zhou.

This section provides a brief description of the methods and materials within this thesis. Information with more details can be found in the attached publication and manuscripts (Paper I, II, III, IV, V).

Animals

The C3H *rd1/rd1* (*rd1*⁷⁹) mouse model, characterized by the fast retinal degeneration due to a mutation in the gene for the beta-subunit of PDE6, as well as their normal healthy wild type (WT⁸⁰) with the background of C3H was used in Paper I, II, and IV. As the function of the rod photoreceptor specific PDE6 is to hydrolyze cGMP, an abnormally high level of cGMP, as well as high activity of the dependent protein kinase G, can be expected in the retinas of the *rd1* model. Therefore, the character of high cGMP levels and PKG activity in rods, and the fast progression of their degeneration, make this animal model an ideal target to study cGMP-PKG downstream signaling in degenerating photoreceptors. The retinas from this model, with the PKG inhibition in organotypic retinal explant culture, were used to generate high throughput data via RNA sequencing and MS. The results of cGMP-PKG-dependent genes and phosphorylation were presented in Paper I and Paper II, respectively. In Paper IV, we picked up a target from the high throughput data, namely CDK1, and studied its role in retinal degeneration with the

same animal models. In Paper III, the C57BL/6J *rd10/rd10* (*rd10*⁸¹) strain, a slower retinal degeneration model with a different mutation in PDE6 was used for the kinome study. In Paper IV, *rd10* and another slow degeneration model, namely C3H *rd2/rd2* (*rd2*⁸²), with a mutation in the gene for peripherin 2 (PRPH2), a protein that stabilizes the outer segment of photoreceptors, were used to study whether PKM2 was involved in retinal degeneration. All animal used in this thesis were kept and bred in house under standard white cyclic lighting, with free access to food and water, and used irrespective of sex. Day of birth of the animal was considered as postnatal 0 (P0), with the day following this considered as P1 and so on. All animal-related experiments and procedures were approved by Malmö - Lunds djurförsöksetiska nämnd, with the permit reference codes as #M92-15 and 02124/2020.

Organotypic retinal culture

The retinas from the *rd1*, *rd2*, *rd10*, and WT of specific age were used to generate the explants, following the protocol as described before⁴⁶. In brief, mice were euthanized and their eyes were rapidly enucleated and incubated in the R16 medium. This included incubation with 0.12% proteinase K diluted with R16 medium for 15 minutes, and activity of the proteinase K was subsequently blocked by 10% fetal bovine serum with the same dilutor, after which the eyes were rinsed in R16 medium. In a sterile environment under a laminar-flow hood, the retina with the retinal pigment epithelium (RPE) attached was separated from the eyes, with the removal of anterior segment, lens, vitreous, sclera and choroid. The retina was then incised to a four-leaf clover shape and transferred to a culture membrane insert, with the RPE directly facing the insert membrane. Inserts with the explants were put into six-well culture plates with 1.5 mL serum-free R16 medium in each well with supplements⁴⁶. Explants in medium were incubated at 37 °C with a CO₂ level of 5%, and the medium was replaced every second day. Retinas were selected randomly for either treatment or control. In Paper I, the retinal explants from *rd1* and WT of P5 were incubated without adding any compounds for 4 days, then on P9, they were treated with Rp-8-Br-PET-cGMPS or 8-Br-cGMP for 2 days, with the end point equal to P11. The culturing paradigm in this Paper was referred to as P5 (starting point) + 4 (untreated period) + 2 d (treated period). In Paper II and Paper IV, different culturing paradigms were performed, including P5 + 2 + 4 d, and P5 + 4 + 2 d in *rd1* explants from P5, with the treatment of Rp-8-Br-PET-cGMPS for 4 days and 2 days, respectively. One more paradigm was included in these two Papers, such that the *rd1* explants were treated with the same compounds for 2 hours prior to ending up at P11, and without any pharmacological manipulation from the same starting point on P5. In Paper III, the paradigm was referred to as P8 + 2 + 8 d of *rd10* explants with Rp-8-Br-PET-cGMPS. In parallel, the untreated *rd10* and WT

with the same paradigm were used as controls. In Paper IV, the *rd2* and *rd10* retinal explants were treated with TEPP-46 (HY-18657, a PKM2 activator) in a P9 + 2 + 8 d paradigm. The explants were then either used for further high throughput analysis, or target validations via immunostaining, TUNEL or PLA after fixation and cryosection.

Genetic study

RNA sequencing

In Paper I, the RNA of each explant was isolated with the QIAGEN RNEasy Mini Kit, with subsequent concentration and quality measurements by a Qubit 4.0 fluorometer and RNA 6000 Nano Assay in 2100 Bioanalyzer, respectively. The RNA of each explant was then used to construct the libraries following the protocol of TruSeq Stranded mRNA Sample Preparation Guild with slight modifications. Then the RNA samples with a concentration of 1.4 pM were loaded on NextSeq 500 to perform paired-end sequencing.

Sequencing analysis and bioinformatics

The generated FASTQ format files were handled in the bcl2fastq2 software to generate the statistics. After the quality control of the raw data via FastQC, the reads were aligned to a specified reference genome Mouse GRCm38 via the HISAT2 software. Then the alignments of reads were assembled into transcripts via StringTie, following the differentially expressed genes (DEG) analysis via R with the package DESeq2. According to the defined criteria (for details refer to Paper I), we then identified four lists of genes that were differentially expressed under the cGMP-PKG regulation.

To gain more insights into how these genes function in molecular signaling, the gene set enrichment analysis (GSEA)⁸³ and the pathway enrichment analysis, including Kyoto Encyclopedia of Genes (KEGG) and Gene Ontology (GO) were done via the online tool (www.gsea-msigdb.org/gsea/index.jsp) and The Database for Annotation, Visualization and Integrated Discovery (DAVID v6.8)⁸⁴ respectively.

Reverse transcription-polymerase chain reaction (RT-PCR)

As the issue of a false positive rate is concerned in RNA sequencing⁸⁵, we selected 6 photoreceptor-specific expressed genes among the DEG identified previously for further validation. The RNA from the explants was used to run RT-PCR. In brief,

the extracted RNA was reversed into cDNA via iScript Reverse Transcription Supermix for RT-qPCR kit. Then the cDNA amplification and quantification were done via a C1000 Thermal Cycler and Bio-Rad CFX Manager 2.0 software.

Proteomic study

Phosphoproteome

In Paper II and Paper III, proteins from explants with or without treatment were extracted after explant homogenization and precipitation of the soluble fraction. The protein was then digested by trypsin, followed by the extraction of peptides. The next step was phosphorylated peptide enrichment, following the instruction of the Fe-NTA Phosphopeptide Enrichment Kit. As the phosphopeptides are more difficult to detect than their unphosphorylated counterparts in MS, the phosphopeptide enrichment in advance of MS can therefore increase the sensitivity of the phosphorylation detection, and avoid the risk of losing valuable phosphorylated proteins due to the low detection rate⁸⁶. The enriched peptide solution was then injected into the LCMS, where they were concentrated on a pre-column (C18) and eluted depending on their hydrophobicity. The Orbitrap Fusion was used to run the MS survey scan and operated in the positive data-dependent acquisition (DDA) mode. According to the full scan MS, the most intense ions were applied for fragmentation in the MS2, i.e. the second MS. Here the ions were run through the quadrupole before being detected in the Orbitrap. The raw data from the MS2 were analyzed with Proteome Discovery™ Software⁸⁷.

The MS results were processed via the Perseus software and further bioinformatic analysis of these processed data was done via the web-based tool Phosphomatics^{87,88} (<https://phosphomatics.com>). In this method, one can expect to identify the series of cGMP-PKG-dependent substrates and the associated kinases that were regulated by this system.

Kinome study

In Paper III, we performed the kinome study with explants with the same paradigm mentioned previously. This microarray-based measurement targets 142 serine/threonine containing immobilized peptides of interest with expected high specificity for given kinases. Therefore, the comparison and combination of results from this kinome study and the previously described phosphoproteomic approach may provide even more valuable insights into cGMP-PKG downstream signaling. In general, the protein extraction of explants was done after the explant samples were lysed, homogenized, centrifuged, and after which the soluble fraction was

collected. To detect the active serine/threonine kinases (STK) in the various samples the same amount of protein, 0.25 µg, from each explant was put together with relevant antibodies and ATP in a protein kinase buffer. Then the assay mix was added to PamChip wells in the PamStation12® system to initiate the measurement. The kinases of the retinal lysate then acted on the immobilized peptides on the chip, after which the potential phosphorylation of these peptides was assessed with the added site specific anti-phospho-serine/threonine antibodies. A fluorescent secondary antibody towards the primary antibodies was then used to indirectly evaluate the kinase activities of the samples, in that the signal intensity of each peptide was captured and quantified by BioNavigator® software version 6.3.67.0. With further upstream kinase analysis in BioNavigator, one can identify the phosphorylations among the 142 peptides that were related to the cGMP-PKG system, and also predict the potential kinases integrated into this network.

TUNEL Assay

TUNEL, known as Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling was applied in the Paper I, III, IV, V. TUNEL is an assay for localization of DNA fragmentation in situ to detect cell death, including apoptosis and necrosis. The general principle of this method is to apply the TdT to attach deoxynucleotides, which are tagged with a fluorochrome, to the 3'-hydroxyl terminus of DNA breaks. In brief, the sections of retinal tissues or explants were fixed with paraformaldehyde in advance. Then the sections were incubated with the TdT reaction mix for 45 minutes. After rinsing and mounting, the TUNEL signal was visualized in Zeiss Axio Imager M2 microscope, and further analysis of the images done via the Zen (2.5) blue edition software (Zeiss Zen software).

Immunostaining

Immunostaining was applied in all Papers in this thesis, since for several of the questions it is advantageous and necessary to detect the protein expression and localization at the cellular level in retinas with multiple layers. In brief, the sections from retinal tissue or explant were fixed with paraformaldehyde in advance. Prior to the staining the sections are blocked with a buffer containing 1% bovine serum albumin (BSA) to avoid any unspecific binding from the antibodies used. Next, the sections are incubated with the primary antibody overnight in a 2-8 °C environment, where the antibody can bind to the target protein. After rinsing the section to wash away the free antibody without target binding, the sections are incubated with a suitable secondary antibody conjugated with varied fluorophores for 45 minutes. After mounting, the target protein was visualized in Zeiss Axio Imager M2 microscope, and the area of interest within the retina analyzed with the aid of Zen (2.5) blue edition software (Zeiss Zen software).

Proximity Ligation Assay

In Paper II, the proximity ligation assay (PLA) was used to evaluate the RAF1 proto-oncogene (RAF1) phosphorylation in retinal degeneration, a kinase we selected for further validation after the phosphoproteomic/MS study. Briefly, antibodies against RAF1 and phospho-serine from different species were used on sections as above, after which secondary antibodies to the two primary antibodies were added. The secondary antibodies each have a so called plus- or minus-DNA oligonucleotide coupled to them (plus on the one secondary, and minus on the other), and when these oligonucleotides are in proximity (<40 nm), they can be used for a hybridization reaction, that initiates the formation of a circularized DNA molecule. After the subsequent amplification via the associated polymerase, the fluorescent signal of the amplified circularized DNA molecule can be visualized in Zeiss Axio Imager M2 microscope. According to this method with antibodies against a specific protein and phosphoserine, the phosphorylation of a target protein can be detected since a close proximity is highly indicative of that the phospho-serine is within the protein of interest, in this case RAF1. However, site-specific information of the phosphorylation is not available.

Results

In this section, I describe briefly the findings in each Paper/manuscript. For the results in detail, please refer to published Papers I and II, and the manuscripts that relate to Papers III to V, which all can be found in the book version of this thesis, or can be provided by Jiaming Zhou.

Paper I: cGMP-PKG dependent transcriptome in normal and degenerating retinas: Novel insights into the retinitis pigmentosa pathology

In this Paper, we manipulated the cGMP-PKG system in a pharmacological way, such that we added an inhibitory cGMP analog to retinas from the *rdl* model with expected high cGMP-PKG activity levels, and in parallel an activatory cGMP analog to normal healthy WT retinas during organotypic retinal culture. It is known that cGMP-PKG regulates several genes in other systems^{68,69}, however, its role as a genetic regulator in retinas is unclear. In this study, lower PKG activities in *rdl* retinas and higher PKG activities in WT can be expected after the treatment, compared to their untreated controls, respectively. This expectation was confirmed by immunostaining of phosphorylated vasodilator-stimulated phosphoprotein (pVASP), a known PKG substrate¹¹⁰, in histological sections from the explant cultural paradigm. In the retinal explant, and specifically in ONL from *rdl* with PKG inhibition, we observed a lower pVASP level than in the untreated peers, while higher pVASP was seen in WT with PKG activation. Another readout, TUNEL, with the function to evaluate photoreceptor death in the same ONL, showed a higher number of TUNEL-positive cells in WT with PKG activation than their counterparts without treatment, while no difference was noticed in *rdl* with PKG inhibition. These results indicated that the cGMP-PKG can be manipulated in a pharmacological way appropriately during retinal culture. Retinal explants from the same organotypic retinal culture paradigm were used to run the RNA sequencing, in order to study the cGMP-PKG-dependent transcriptome. With the predefined selective criteria, we identified a series of genes that were under the regulation of cGMP-PKG according to the data mining from the sequencing results. Among the cGMP-PKG-dependent genes identified from RNA sequencing, we selected 6

known photoreceptor-specific genes, namely *Cngb1*, *Pde6g*, *Prcd*, *Prph2*, *Rom1* and *Guk1* for further validation via qPCR, and found that qPCR results were largely consistent with the sequencing (Figure 2). Further bioinformatic analysis regarding the pathway enrichment of the identified genes was performed with the online tool The Database for Annotation, Visualization and Integrated Discovery (DAVID) and Gene Set Enrichment Analysis (GSEA). We found that the cGMP-PKG system negatively regulates oxidative phosphorylation and mitochondrial pathways at the transcriptional level, which may affect retinal degeneration. These results also suggested that the cGMP-PKG system affects the photoreceptor-specific genes, whose mutations lead to RP.

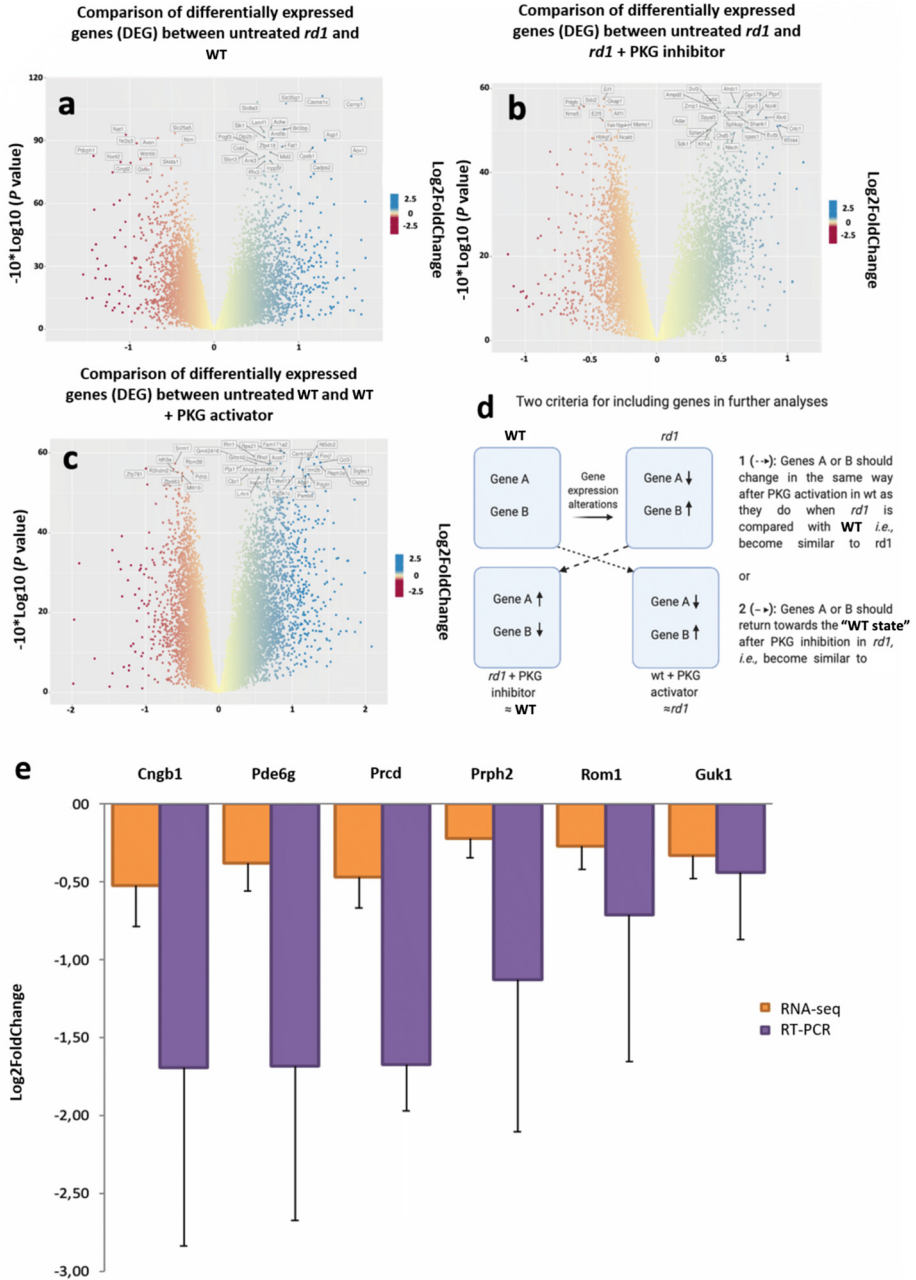


Figure 2: Volcano plots for three comparisons, selection criteria, and an RT-PCR test.

a) Comparison of DEG between untreated *rd1* and WT; b) comparison of DEG between *rd1* and *rd1* treated with inhibitor; c) Comparison of DEG between WT and WT treated with activator; 5 biological replicates of *rd1* explants with PKG inhibitor and their untreated counterparts, respectively and 3 biological replicates of WT explants with PKG activator and the same number of their untreated control were included in the RNA sequencing; d) selective criteria for genes to be accepted as candidate cGMP-PKG regulated genes: Criterion 1: A gene in inhibitor treated *rd1* explants should show a differential expression to untreated *rd1* explants, that is similar to the differential expression of that gene between untreated *rd1* explants and untreated WT explants, but in the opposite direction. Thus, *rd1* genes that are different from WT should be “altered back” to WT by the inhibitor treatment. Criterion 2: A gene in activator treated WT explants should show a differential expression to untreated WT explants, that is similar to the differential expression of that gene between untreated WT explants and untreated *rd1* explants. Thus, WT genes that are different from *rd1* should after treatment be similar to *rd1*; e) Verification of RNA-seq via RT-PCR. In the comparison between WT and WT treated with 8-Br-cGMP, the orange and purple bars represent fold change in RNA-seq and RT-PCR, respectively. Purple bars (RT-PCR) represent mean \pm S.D., $n = 2-3$ (biological replicates). DEG = Differentially expressed genes. This figure is reprinted from Zhou et.al.¹²⁹

Paper II: The phosphoproteome of the degenerating *rd1* retina changes after protein kinase G inhibition

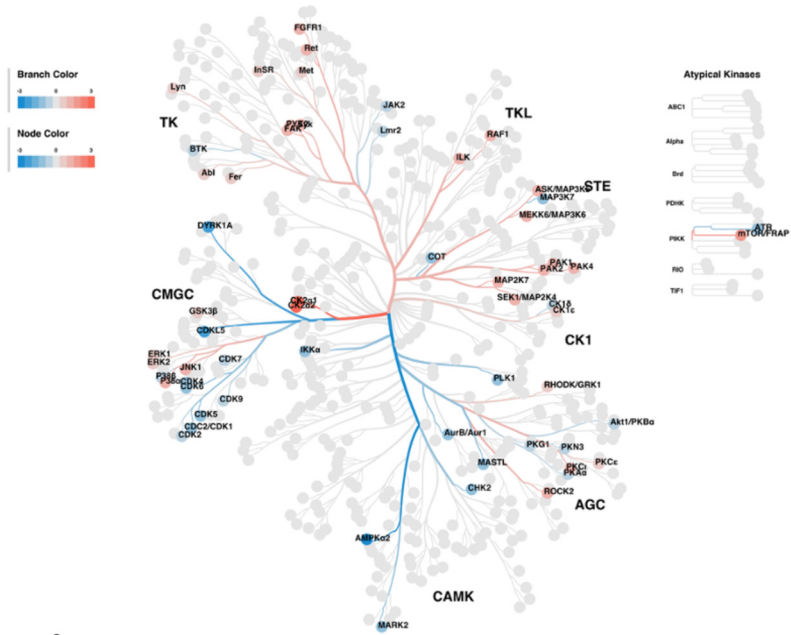
In our understanding, the main function of PKG is to phosphorylate its substrates, and therefore we hypothesize that the abnormally high cGMP-PKG activity causes retinal degeneration via over-phosphorylation, of yet unknown substrates. This Paper applied a phosphoproteomic approach via the combination of phosphopeptide enrichment and MS to gain more insights into cGMP-PKG downstream signaling in degenerating photoreceptors. We inhibited the cGMP-PKG activities in *rd1* retinas for different lengths of time with our previously used manipulation system, in which an inhibitory cGMP analog was added during organotypic retinal explant culture for 2 hours or 4 days towards the end of the culturing period. The retinal explants with PKG inhibition, complemented with their untreated controls, were used to perform the phosphoproteomic study. In this MS-based study, we identified 17915 phosphorylated sites and 3405 proteins, among which 483 sites of 182 proteins and 628 of 174 proteins were altered significantly in retinal explants with PKG inhibition for 2 hours and 4 days, respectively. With these groups of cGMP-PKG-dependent substrates, we predicted a series of kinases that may be integrated into the cGMP-PKG network (Figure 3). This is because these substrates may be regulated by specific kinases to implement diverse molecular functions, therefore the possibility of cGMP-PKG regulating these substrates in an indirect manner via other kinases has to be considered.

Apart from providing a general overview of the possible PKG substrates in the retina, we also among these kinases with potential but unclassified function in retinal degeneration picked up RAF1 proto-oncogene (RAF1) for further analysis. In a comparison of the RAF1 expression of ONL between *rd1* and WT we found that RAF1 was predominantly expressed in the ONL of retinal sections, according

to the immunostaining data. Moreover, a comparison of the RAF1 expression in photoreceptors as such between *rdl* and WT revealed a higher RAF1 expression in *rdl* (Figure 4). To study if any relations between the cGMP-PKG system and RAF1 exist, we immunostained for RAF1 in retinal explants with PKG inhibition from *rdl*, observing that the RAF1 expression was higher in the ONL after PKG inhibition, regardless of the different lengths of treatment.

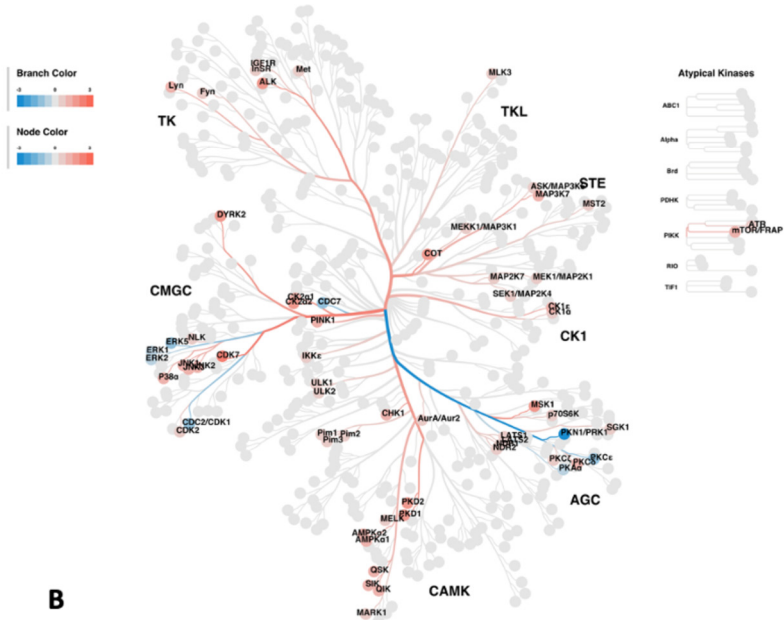
We in addition studied the RAF1 phosphorylation with the so-called proximity ligation assay (PLA) technique and found a higher extent of RAF1 phosphorylation in ONL from *rdl* than in WT. We also observed that RAF1 phosphorylation was higher in *rdl* retinal explant being treated with PKG inhibitor for 2 hours, while no difference was noticed after 4 days of treatment with the same strain. These results indicate the possibility that RAF1 may work as a cGMP-PKG substrate and function in degenerating photoreceptors in an as yet undiscovered mechanism.

***rd1* explants with 2 hours of PKG inhibition vs untreated *rd1* explants**



A

***rd1* explants with 4 days of PKG inhibition vs untreated *rd1* explants**



B

Figure 3: Potential altered kinases are visualized as a kinome phylogenetic tree.

The branch and node color are encoded by Fold Change, with values < 0 (in blue) and > 0 (in red) representing kinase activity as decreased or increased, respectively, in *rd1* explants with PKG inhibition than untreated *rd1* explants. A: kinome phylogenetic tree with potential kinases altered in *rd1* with PKG inhibition for 2 hours. B: kinome phylogenetic tree with potential kinases altered in *rd1* with PKG inhibition for 4 days.

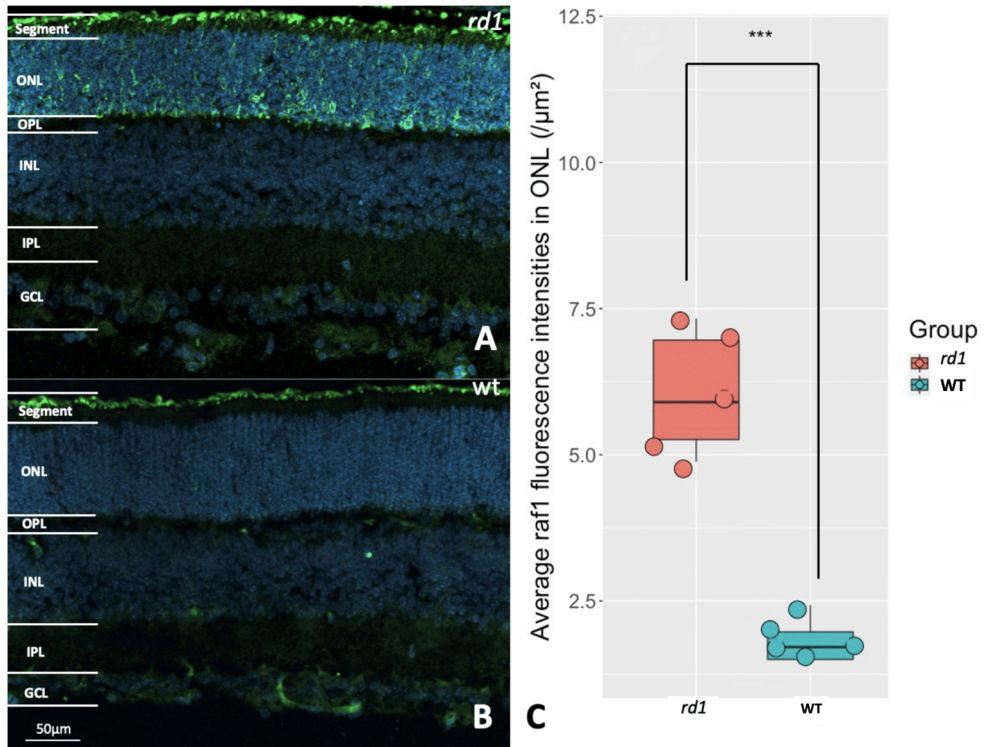


Figure 4: Evaluation of RAF1 expression in *rd1* and WT retinas.

A: Immunostaining of RAF1 (green) in a P11 *rd1* retina. B: Immunostaining of RAF1 (green) in a P11 WT retina. DAPI (blue) was used as nuclear counterstain. C: The box chart shows the comparison of RAF1 fluorescence intensities within the outer nuclear layer (ONL) between *rd1* and WT (n=5), ***p < 0.001.

Paper III: Integrative kinase activity profiling and phosphoproteomics of retinal explants during cGMP dependent retinal degeneration

We investigated the cGMP-PKG-dependent kinome and phosphoproteome via a microarray-based technique and mass MS, using retinal explants from a different rd model (*rd10*) and a culturing paradigm involving a longer culturing period. Using TUNEL to evaluate the photoreceptor death, we found lower TUNEL-positive cells after treatment with PKG inhibition in explants from the *rd10* model compared to untreated such explants. This was complemented with the observation of lower TUNEL-positive cells in untreated WT counterparts in the same culturing paradigm. This provided validation of the neuroprotective effect of PKG inhibition in photoreceptor degeneration, and also of that the rationale for our pharmacological method to manipulate the cGMP-PKG was appropriate for further investigation.

The explants from the same culturing paradigm were then used to perform microarray using specifically designed kinase substrate chips, as well as phosphopeptide enrichment and MS. According to the microarray data, we found that phosphorylation of seven peptides, namely, CAC1C_1974_1986, ESR1_160_172, PLM_76_88, CREB1_126_138, PTK6_436_448, TOP2A_1463_1475, RBL2_655_667, CGHB_109_121, and STK_283_295, decreased in *rd10* with PKG inhibition, whereas increased phosphorylation of the peptide H2B1B_27_40 was noticed. With the upstream kinase analysis, we predicted that Ca²⁺/calmodulin-stimulated kinase (CaMK), casein kinase 1 (CK1) and protein kinase A, G, and C (AGC) families, such as CaMK4, PKA α , CK1 ϵ , CDKL1, PKD1, PKG1, PKG2 to have reduced activity in *rd10* explants with PKG inhibition.

For the MS study, we found that 85 phosphorylated sites of 50 proteins were altered in *rd10* treated with PKG inhibitor. In this line of study, we predicted a series of kinases, including calcium/calmodulin-dependent protein kinase II alpha (CaMK2 α), mitogen-activated protein kinase 14 (MAPK14) and tyrosine-protein kinase ZAP-70 (ZAP70), as well as several others to have decreased activities with PKG inhibition. The kinases with increased activities after the treatment were also predicted, such as ribosomal protein S6 kinase beta-1 (RPS6KB1 or p70S6 kinase), cyclin-dependent kinase 2 (CDK2), and FYN proto-oncogene (FYN) and more.

Among the targets identified, we selected several proteins (CREB, CaMK2 and CaMK4), corresponding to the peptides that were differentially phosphorylated, for further investigation. According to the immunostaining of these targets in retinal sections, we found a significantly higher CaMK2 phosphorylation in ONL from *rd10* than from WT, while the expression of CaMK2 was also lower in a similar comparison, although statistical significance was not attained. Similarly, the phosphorylation of CREB was also lower in ONL from *rd10* than from WT. By

contrast, the CaMK4 expression as well as the phosphorylation of this kinase were higher in ONL from WT. We also compared the localization of these proteins in photoreceptor segments between *rd10* and WT, and the results were consistent with the ones on ONL (Figure 5).

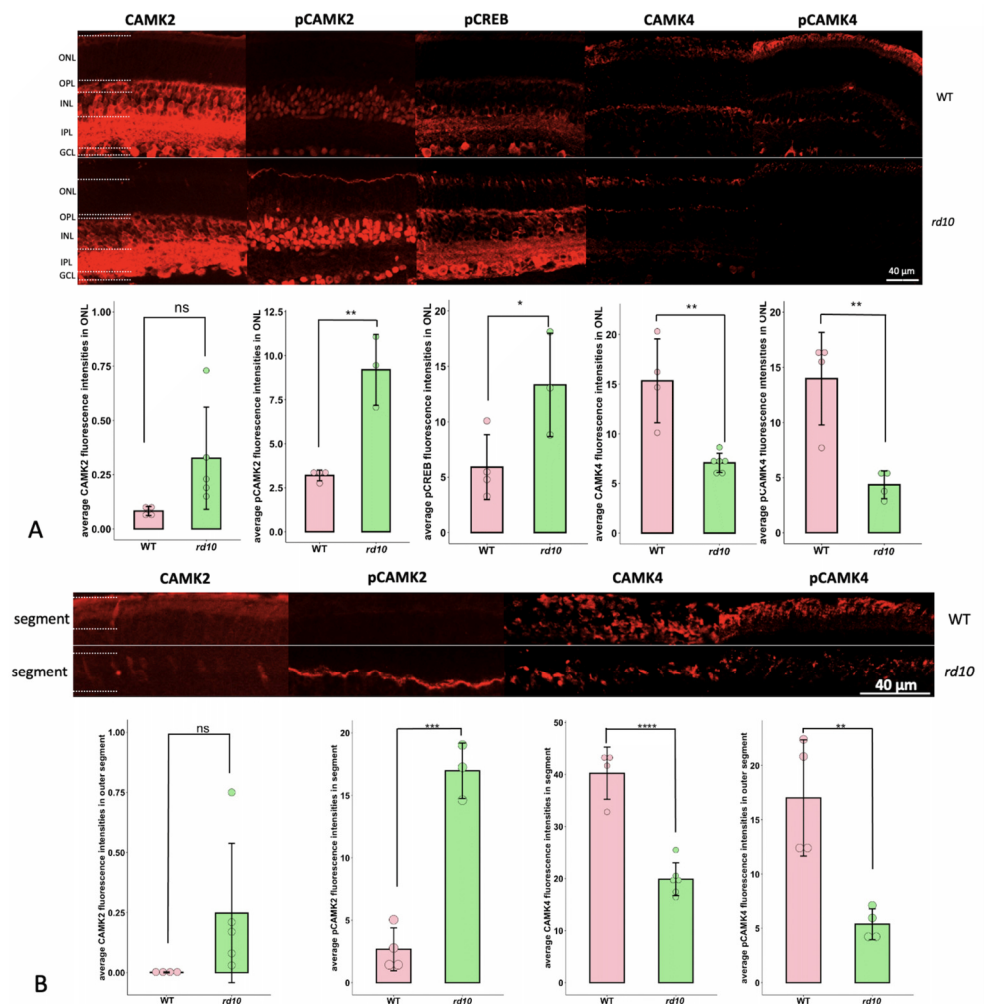


Figure 5: Presence and localization of PKG target proteins in WT and *rd10* retina by immunofluorescence.

A: The panels show retinal cross-sections derived from WT (top) and *rd10* (bottom) P18 mice and stained with antibodies for CaMK2, pCaMK2, CaMK4, pCaMK4 or pCREB. The fluorescence intensities were calculated as arbitrary fluorescence units/ μm^2 in the area of interest. B: The panels show staining of CaMK2, pCaMK2, CaMK4 and pCaMK4 in retinal segments from WT and *rd10* P18 mice. The fluorescence intensities were calculated as arbitrary fluorescence units/ μm^2 in the area of interest. The fluorescence was adjusted with overexposure in the images for presentation purposes. ONL: Outer nuclear layer, OPL: Outer plexiform layer, INL: Inner nuclear layer, IPL: Inner plexiform layer, and GCL: Ganglion cell layer.

Paper IV: A potential role of cyclic dependent kinase 1 (CDK1) in late stage of retinal degeneration

In this Paper, we showed that the cyclic dependent kinase 1 (CDK1) may function in a late stage of retinal degeneration. We noticed CDK1 expression in photoreceptors undergoing degeneration via immunostaining for CDK1 in retinal sections, specifically in ONL from the *rdl* model. We then provided more details regarding whether the CDK1 was related to photoreceptor death. To achieve this, we co-stained for CDK1 and acetylated lysine or TUNEL, two known markers for the early and late stages of cell death, respectively, in photoreceptors. Photoreceptors have previously been shown to lack acetylated lysine when in their early stages of degeneration. We found a very limited overlap between CDK⁺ and acetylated lysine-negative cells, while the CDK⁺ and TUNEL overlap was extensive. This indicated that CDK1 mainly plays a role in the photoreceptors when the degeneration progresses to a later stage (Figure 6).

We were also interested to investigate if CDK1 was related to the cGMP-PKG system, and therefore inhibited the cGMP-PKG system by adding an inhibitory cGMP analogue for various treatment lengths, i.e. either the final 2 hours, 2 days or 4 days during organotypic *rdl* retinal explant culture. Compared to the untreated counterparts, we could not find any alteration of CDK1 expression after 2 h of PKG inhibition, while it was evidently less expressed after 2 days and 4 days of treatment. According to these data, we propose that CDK1 may act as a cGMP-PKG downstream effector, at least related to this system in an indirect way.

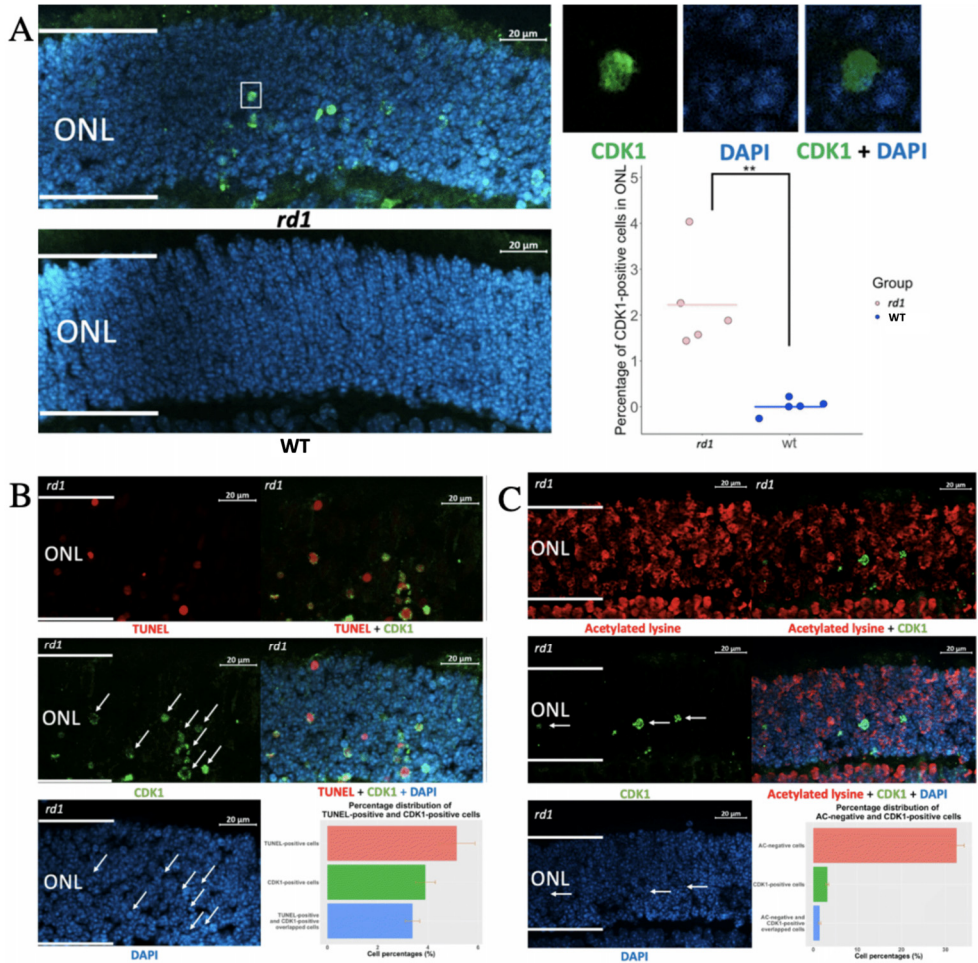


Figure 6: Evaluation of CDK1 expression within the ONL.

(A) Immunostaining of CDK1 (green) within the ONL of *rd1* and WT strains at P11. The dot chart shows the comparison of CDK1-positive cells within ONL between *rd1* and WT (n = 5); (B) Co-staining of CDK1 (green) and TUNEL (red) within ONL from *rd1* retinas, with bar chart showing the distribution of TUNEL-positive and CDK1-positive cells (n = 5). (C) Co-staining of CDK1 (green) and acetylated lysine (AC, red) within the ONL from *rd1* retinas, with bar chart showing the distribution of CDK1+ cells and AC-negative cells (n = 5). DAPI (blue) was used as nuclear counterstain. For graphs, bars represent mean \pm S.D., ** p < 0.01. ONL = Outer nuclear layer. Arrows point to the CDK1-positive cells and associated DAPI-positive cells. This figure is reprinted from Zhou et.al 2022¹³⁰.

Paper V: Pyruvate kinase 2, an energy metabolism related enzyme, may have a neuroprotective function in retinal degeneration

In this study, we further investigated the role of one target we identified from the previous high throughput data, namely PKM2, during retinal degeneration. We manipulated the PKM2 activities within the photoreceptors in two other rd models, namely *rd2* and *rd10*, by adding TEPP-46 (a PKM2 activator) during the organotypic retinal explant culture. Using TUNEL to evaluate photoreceptor death, we could not observe any difference in *rd2* explants with PKM2 activation compared to their untreated controls, while by contrast, the TUNEL-positive photoreceptors decreased in *rd10* retinal explants after being treated with PKM2 activator (Figure 7).

We then explored how PKM2 appears in degenerating photoreceptors. We immunostained for PKM2 in retinal tissues from *rd2*, *rd10* and WT in P9 and P19, and found a lower PKM2 expression of photoreceptors in *rd2*, as well as in *rd10*, than in WT on P9. On P19, we saw a similar lower PKM2 expression in *rd2* than WT, while higher PKM2 expression was seen in *rd10* than in WT.

To provide more details regarding how PKM2 downstream activities were affected in degenerating photoreceptors, we performed more investigation on a known PKM2 target, namely glucose transporter-1 (Glut1), in the same retinal tissue and culturing paradigm. We noticed a lower Glut1 expression in *rd2* and *rd10* than in their WT controls at P9, while no difference was observed in P19 between these two rd models with WT. For *rd2* with PKM2 activation, we observed that PKM2 activation led to higher Glut1 expression in segments in *rd2* than in their untreated peers. For *rd10*, no significant difference in Glut1 expression was noticed. To sum up, this study showed that PKM2 activation can alleviate photoreceptor cell death in the *rd10* models, while it cannot generate neuroprotective effects in the *rd2* models, at least during the conditions used here. The PKM2 may have a neuroprotective role during retinal degeneration as a lower expression of this kinase was seen in these two *rd* models in P9 than in their WT counterparts.

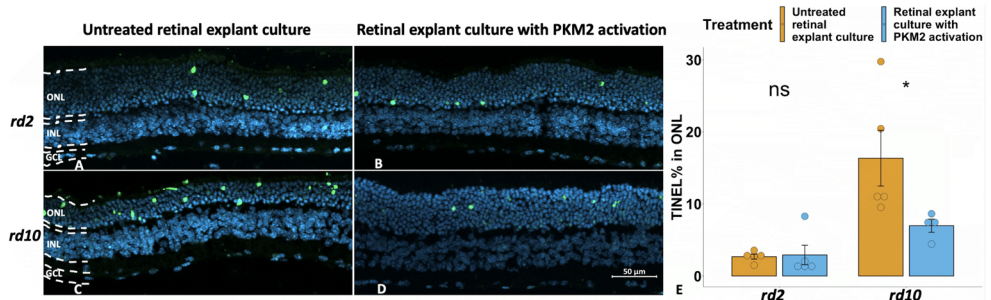


Figure 7: activation of PKM2 showed a decreased number of dying photoreceptors in *rd10* explants, while the same treatment did not change these parameters in *rd2* explants.

TUNEL-positive cells are shown in green, and DAPI (blue) was used as nuclear counterstain. A: *rd2* untreated control; B: *rd2* treated with TEPP-46 (PKM2 activator); C: *rd10* untreated control; D: *rd10* treated with TEPP-46; E: Bar chart summarizing the TUNEL-positive ONL cell counts of the different experimental groups, respectively. ONL=Outer nuclear layer; INL=Inner nuclear layer; GCL=Ganglion cell layer. N=4-5 biological

Discussion

As the cGMP-PKG system is regarded as one of the prime effectors to drive retinal degeneration, as discussed above, it would be informative to explore the downstream signaling for therapeutic purposes. This thesis presented potential cGMP-PKG-dependent genes, according to the manipulation of PKG activities followed by RNA sequencing analysis (Paper I). We provided insights regarding how the cGMP-PKG system affects the photoreceptors' wellbeing via its connections with several photoreceptor-specific expressed genes. Notably, among these genetic targets, the expression of peripherin 2 (Prph2), and its homolog rod outer segment membrane protein 1 (ROM1) became lower after PKG activation in WT, which corresponded with the untreated WT/*rdl* difference. As these two genes are specifically expressed in photoreceptors, and whose assembly complex is essential for the maintenance of the outer segment structure⁹⁰, the decrease in Prph2 and ROM1 expression could therefore affect the stability and add to the burden of photoreceptors with high cGMP levels. It is well known that gene mutations or protein defects of these two targets lead to two types of RP^{82,91}. Other genes, whose mutants cause RP-disease phenotypes, including Cngb1⁹², Pde6g⁹³, Prcd⁹⁴, as well as Guk1⁹⁵, which interacts with the product of such an RP causing gene (RD3), also became lower expressed in WT with PKG activation in our study. These data depict the scenes of a retinal disorder originating in photoreceptors with abnormally high cGMP levels and PKG activity.

Though not photoreceptor-specifically expressed, some cGMP-PKG downstream genetic targets are of particular interest as they have been linked to photoreceptor death. Itr3, with the function in photoreceptors to release intracellular Ca²⁺⁹⁶, had a lower expression in *rdl* after PKG inhibition. As a disturbed Ca²⁺ influx apparently causes photoreceptor cell death⁹⁷, it is reasonable to deduce a neuroprotective effect during retinal degeneration of PKG inhibition via negative regulation of Itr3 expression. Also in the same treatment, we noticed a higher expression of Vdac2, known as an anti-apoptotic factor that can inhibit the pro-apoptotic factor BAK⁹⁸. This may promote the survival of photoreceptors via a better withstanding of the apoptosis process. However, we also observed that when PKG was activated in WT, Itr3 and Vdac2 became higher and lower expressed, respectively. Thus, it is possible that the detected genes were changed under PKG manipulation and regulated by a combination of several other factors.

It is worth mentioning that the cGMP-PKG system may not affect retinal degeneration via a single target or pathway, and thus applying the pathway enrichment analysis with the genes identified may provide more insights into cGMP-PKG functions. When this is done, the results suggested cGMP-PKG system to be a suppressor of processes related to mitochondrial activity, including oxidative phosphorylation, and the abnormally high level of cGMP-PKG levels in degenerating photoreceptors may therefore negatively affect energy metabolism via lower expression of associated genes. Given that photoreceptors are one of the highest metabolically active cells⁹⁹, they are thus very vulnerable to energy depletion and mitochondrial failure^{100,101}. In photoreceptors, the required energy for consumption is several times higher in the dark than in light⁹⁹, with a possible contribution of ion influx through the open CNG channel¹⁶⁴. As a known cGMP effector, the CNG is expected to be open in RP, where cGMP levels are high. Therefore, a high amount of ATP, whose additional production is provided by oxidative phosphorylation apart from glycolysis¹⁰², would be needed to maintain the ion level in such situations. This could make the RP photoreceptors more energetically vulnerable at high cGMP levels. More evidence was recently provided to prove the detrimental effects of metabolic dysregulation on retinal degeneration, with the reported starvation of photoreceptors and vision loss in a pig RP model, due to the disturbance of glucose transport from RPE to photoreceptors¹⁰³. There have also been clinical reports of patients with vision problems due to a deficiency in glucose transporter type 1 (Glut1), which functions to carry glucose through the blood-brain barrier¹⁰⁴ (more details on the role of Glut1 in degeneration are mentioned below). The possibility that cGMP-PKG affects energy metabolism by suppressing the oxidative phosphorylation pathway can therefore be proposed in this thesis.

Another essential ATP supplier via oxidative phosphorylation to satisfy the high metabolic requirement in photoreceptors are fatty acids, which was recently recognized¹⁰⁵. Studies on one lipid metabolism-related receptor on photoreceptors, namely very low-density lipoprotein receptor (VLDLR), with the function to increase lipid uptake into cells, provided revealing insights into this topic¹⁰⁶. Specifically, applying a genetic modification technique to knock out the VLDLR in a mouse model showed a decreased mitochondrial supply within photoreceptors¹⁰⁶. Similarly, the deletion of peroxisome proliferator-activated receptor-alpha (PPAR α) in another strain, which acts as a regulator of fatty acid oxidation, led to poor photoreceptor survival¹⁰⁶⁷. Our sequencing data found that one lipid metabolism-related target, sterol regulatory element-binding transcription factor 1 (Srebf1)¹⁰⁸, with known neuroprotective effects¹⁰⁹, was lower expressed after PKG activation in WT. Hence, a detrimental effect of lipid metabolism dysregulation to photoreceptors by cGMP-PKG overactivation should not be ignored.

As the main function of PKG is to phosphorylate its substrates, it is essential to acquire more comprehension of cGMP-PKG downstream signaling at the

phosphoproteome level. In Paper II and Paper III, we identified a series of potential cGMP-PKG-dependent phosphoproteins. In our hypothesis that the degeneration is driven by the hyperactivation of PKG, then the proteins that showed decreased phosphorylation in RP models under PKG inhibition could be expected to have a pathologic involvement. Interestingly, according to the microarray-based measurement, we noticed reduced phosphorylation at the serine 239 position of VASP, a known PKG substrate¹¹⁰, under PKG inhibition in RD retinal explants. This stands in line with the immunostaining results with the same target in Paper I, and proves the consistency of lower PKG activity under the inhibitory PKG manipulation. Among the identified reduced phosphorylated sites from the MS-centric detection, we found that phosducin¹¹¹ with lower phosphorylation appeared in two RP strains treated with PKG inhibitor. This is consistent with the fact that higher phosphorylation of phosducin was reported in the degenerating photoreceptors in *rd1*¹¹². Our data thus proposed the possibility of PKG being responsible to increase phosducin phosphorylation that contributes to photoreceptor degeneration.

While cGMP-PKG is expected to function via the phosphorylation of its substrates, we could not exclude the possibility of this system regulating the downstream targets in an indirect manner. That is, the cGMP-PKG may affect other kinases that indirectly work on the substrates. With the aid of bioanalysis, we predicted the potential kinases that may be regulated by cGMP-PKG. Among these potentially affected kinases, RAF1, CaMK2 and CaMK4 were of particular interest for further investigation.

For the RAF1, our current understandings are in line with its involvement in the well-known RAS/RAF1/MAPK/ERK pathway, that has multi-biological functions¹¹³. In the case of retinal tissue, RAF1 is essential for eye development¹¹⁴ and promotes diabetic retinopathy development¹¹⁵ in physiological and pathological scenarios, respectively. The thesis presents novel insights regarding the RAF1 expression in retinal tissue, including that the RAF1 is a photoreceptor-specific kinase with the observation of predominant expression of RAF1 in the photoreceptor segments. When combining the results of higher RAF1 expression in ONL from *rd1* than from the WT counterpart, this indicates that RAF1 may participate in retinal degeneration. Further confirmation with respect to RAF1 being regulated by cGMP-PKG was provided in *rd1* explants with PKG inhibition during culturing. Our data showed more RAF1 phosphorylation when PKG was inhibited by 2 hours in *rd1* explants in the PLA assay¹¹⁶. Given that PKG inhibition alleviates retinal degeneration, we therefore proposed a neuroprotective effect of RAF1 in degenerating photoreceptors. This may be achieved by regulation and phosphorylation of its substrate, termed Mammalian Ste20-like kinases (MSTs), with a recognized role to induce neuronal cell death¹¹⁷. Specifically, the neuron death induced by MST would be suppressed and reversed when it is phosphorylated by RAF1¹¹⁸. Thus, the RAF1/MST2 complex may have neuroprotective effects

during retinal degeneration. Another possible neuroprotective pathway of RAF1 is to inhibit apoptosis signal-regulating kinase-1 (ASK1), in a protein-protein interaction manner¹¹⁹. The rationale is that ASK1 participates in stress-induced neuronal death and the observation of reduced photoreceptor death in the *rd1* model under RNA interference of ASK1¹²⁰. Under these circumstances, RAF1 may act as one of the cGMP-PKG downstream targets that participates in the photoreceptor death process.

The kinase activities of CaMK2 and CaMK4 were also potentially linked to the cGMP-PKG system according to our kinome studies. In general, CaMK2 signaling cascade have been identified as one of the downstream pathways of cGMP-PKG¹²¹, and calmodulin is a major Ca²⁺ binding protein which activates CaMK2¹²². It is known that CaMK2 takes part in cell death, with its reported inhibition to prevent neuronal cell death¹²³. In the immunostaining for CaMK2 in *rd10*, another RP model with high photoreceptor cGMP and likely also PKG activity, a phosphorylated variant of CaMK2, pCaMK2, was seen to have a higher level in photoreceptors, and specifically in segments, than in WT, without any difference in CaMK2 expression. This indicated a higher CaMK2 phosphorylation in degenerating photoreceptors. This goes well with the previous work from the laboratory, showing increased CaMK2 activities, with the readout of its known phosphorylated substrate phosphoducin, in *rd1* than WT¹¹². For CaMK4, the data from the immunostaining showed higher expression and phosphorylation of CaMK4 in segments of photoreceptors in WT than *rd10*. We considered the neuroprotective effects of CaMK4, with the supportive evidence that it promotes neural cellular survival and is decreased in neurodegenerative diseases, such as amyotrophic lateral sclerosis¹²⁴. The likely mechanism for CaMK4 to alleviate the neurodegeneration is to phosphorylate its substrate NF-kappa B and activate the anti-apoptotic gene expression¹²⁵. All in all, these results propose the possibility of the involvement of CaMK2 and CaMK4 in retinal degeneration, as well as a link with cGMP-PKG.

CDK1, whose activities became lower under PKG inhibition in *rd1* explants according to the kinome part of the phosphoproteomic investigation, was further explored in Paper IV with a focus on whether it participates in photoreceptor degeneration and its possible relationship with the cGMP-PKG system. This study provides novel insights into CDK1 expression in retinal degeneration, which had not been previously reported, although some other cell cycle proteins, such as cyclin dependent kinase 2 and cyclin dependent kinase 4, have been investigated in *rd1*¹²⁶. To be more specific, the CDK1 is expressed in degenerating photoreceptors, particularly in the later stage of photoreceptor death. As increased photoreceptor survival was reported in a CDK1 knock-out strain and in an animal experiment with CDK1 inhibition by intravitreal injection, reducing CDK1 is expected to have protective effects in retinal degeneration¹²⁷. Additionally, Paper IV provided clues to the association of cGMP-PKG and CDK1, as lower expression of CDK1 observed after PKG inhibition during retinal explant culture. This is consistent with the kinase

activity prediction in Paper II, that the CDK1 activity became lower soon after PKG was inhibited, with the same animal and culturing protocol applied in both types of experiment. When combining these results, one possible interpretation would be that CDK1 expression was affected by its phosphorylation, which is regulated by cGMP-PKG. A further study, e.g. applying PLA with the anti-CDK1 and anti-phosphoserine antibodies may provide more persuasive evidence, particularly regarding the CDK1 phosphorylation in retinal explants from disease models with PKG inhibition. In addition, one more CDK1 function is to upregulate mitochondrial respiration to initiate the cell cycle, as this process consumes a lot of energy¹²⁸. In Paper I, we stressed that PKG inhibition may positively regulate the oxidative phosphorylation-related pathway to alleviate photoreceptor degeneration. This makes sense as photoreceptors are one of the highest metabolic cells. Hence, as a speculation, the progressively decreased CDK1 expression within photoreceptors after PKG inhibition could be possible because the oxidative stress of photoreceptors was alleviated after PKG inhibition. These data suggest that CDK1 has functions in the late stage of photoreceptor degeneration and is involved in the cGMP-PKG network.

In Paper V, PKM2 was selected for further investigation on whether it participates in retinal degeneration and if it has an association with cGMP-PKG. The rationale is that this kinase is expressed within photoreceptors and that a lower PKM2 expression was observed in WT when PKG was activated in Paper I. Given that Paper I also showed that PKG activation in WT leads to more photoreceptor cell death, the possibility of PKM2 having neuroprotective effects was considered. Therefore, activation of PKM2 was expected to alleviate the retinal degeneration in two diseased models, namely *rd2* and *rd10*, as studied in Paper V.

While applying the TUNEL assay as a cell death marker, the data in retinal explants from *rd10* with PKM2 activation was consistent with our hypothesis. To be more specific, a lower count of TUNEL-positive cells was observed in the explants with treatment than in their untreated peers. However, this was not the case in *rd2* as no difference in cell death profile was noticed after PKM2 treatment. One possible interpretation was that we for both models performed our culturing protocol from P9 to P19, and a high percentage of cell death has been reported around P18 in *rd1063*. For *rd2*, while a peak of cell death was reported at P18, the cell death was also noticed some days later⁶². This may explain the difference in protective effects with the same PKM2 activation between the two RD models, such that a longer treatment may be required to detect the neuroprotective effects in *rd2*.

As the *rd2* and *rd10* models were well documented with high cGMP levels in their photoreceptors, and therefore most likely PKG activities, the PKM2 expression in these two models was lower on P9 than the WT counterparts goes well with the hypothesis of neuroprotective effects of PKM2. The observation of higher PKM2 in *rd10* than in WT, which was not consistent with our hypothesis, may be the result of a compensating effect to counteract the *rd10* degeneration, which is seen by the

clear shrinkage in ONL of *rd10* in P19 compared to P9. For *rd2*, a difference in thickness was not noticed between the same ages, that could indicate that similar compensating effects have not started yet at this point in this strain. This again provided an argument and potential explanation for that PKM2 activation did not have protective effects on *rd2*.

One of the PKM2 targets is Glut1, whose expression was also studied in retinal cross-sections in *rd2* and *rd10*. Similar findings were obtained such that lower Glut1 expression was seen in *rd2* and *rd10* than in their WT peers at P9, while no difference between these three strains at P19. All data combined indicated that PKM2 may function, at least in part, via Glut1 at an early age, which is consistent with the discoveries reported by Rajala et al⁷⁶. However, the correspondingly expressed PKM2 and Glut1 at a later age pointed to the possibility that some downstream signaling leads also to effects that extend beyond Glut1, which deserves further analysis.

All in all, this thesis explored the cGMP-PKG downstream cascade via the manipulation of cGMP-PKG in retinas from retinal degeneration WT models, with the aid of organotypic explant culture technique. A series of genes (Paper I) and substrates (Paper II, III) that were potentially regulated by this system were generated, according to the application of RNA sequencing and MS-based technique, respectively. Among these targets, this thesis picked up several of them, namely RAF1(PaperII), CDK1 (Paper IV), and PKM2 (Paper V), and studied whether they play a role during retinal degeneration. The present data suggested that RAF1 and PKM2 may have neuroprotective effects on degenerating photoreceptors in an unclarified mechanism, while CDK1 appears to function in the later stage of retinal degeneration. These results gain insights into potential novel molecular targets for the treatment development purpose for RP.

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