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Final Degree Thesis

Subcellular localization of blindness-causative protein RD3 assessed by a genetic approach

Academic director: Javier Ugarte Chicote, PhD

Associated teacher of biochemistry and biotechnology department, URV

Professional director: Ana Méndez Zunzunegui, PhD

Department of Physiological Sciences, UB

Cellular and Molecular Basis of Sensory Disorders, IDIBELL

Author: Alba Soler Boronat (alba.solerb@estudiants.urv.cat)

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CENTER DATES

The laboratory of Ana Méndez Zunzunegui is focused on understanding the cellular and molecular mechanisms by which photoreceptor cells of the retina respond to light, and how gene defects at this level lead to blindness, with the ultimate goal of identifying new therapeutic targets for inherited retinal dystrophies. Ana Méndez Zunzunegui is a professor at the Department of Physiological Sciences at the University of Barcelona (UB) School of Medicine, and a member of the Cellular and Molecular Neurobiology Group of the Bellvitge Biomedical Research Institute (IDIBELL).

One of the objectives in the lab is to identify the initial trigger of retinal degeneration and the signaling pathways that accompany the progression of the disease for different forms of inherited blindness, in order to identify novel therapeutic targets.

In particular the project that I had been working on, focuses on the characterization of the RD3 protein, which is a protein that has been linked to a severe form of congenital blindness, Leber Congenital Amaurosis 12, and reported to be essential for the stability and trafficking of retinal guanylate cyclase, a central protein in photoreceptor cell physiology. Mutation K87E in RD3 leads to retinitis pigmentosa, and we here generated and analyzed transient transgenic mice expressing wild type and mutant RD3 in order to study the effect of the mutation in living photoreceptors.

ABSTRACT

Light transduction to an electrical signal at photoreceptor cells of the retina constitutes the first step in vision. Gene mutations causing functional defects at this level lead to blindness. Mutations in RD3, a protein required for the stability and trafficking of retinal guanylate cyclase that synthesizes cGMP, the key signaling molecule in phototransduction, are linked to severe forms of

blindness. It is not yet clear how mutations in RD3 compromise the protein function and photoreceptor cell physiology, leading to retinal degeneration. Controversial results from different laboratories have made the subcellular localization of RD3 a key unresolved question. We here have assessed RD3 subcellular localization in rods by making transient transgenic mice that express RD3.V5 in rod photoreceptors of rd3 mice, a naturally-occurring mouse model of RD3 loss of function. By using antibodies to the V5 tag, we show that RD3 localizes mainly to the inner segment compartment of rods, and is largely excluded from the outer segment. Importantly, its expression restores normal RetGC expression and trafficking to the outer segment. Expression of transgenic K87E/RD3.V5, a mutant that leads to retinitis pigmentosa, reveals that mutant RD3 distributes as the control protein, but fails to fully rescue RetGC trafficking to the outer segment. In K87E/RD3.V5 transfected cells, a fraction of RetGC miss localizes at the cell proximal compartments. Therefore we conclude that the retinitis pigmentosa-causative mutation K87E leads to an intermediate phenotype between complete loss-of-function mutations linked to LCA12, and the wild type protein in a physiological context. Our results explain the mild phenotype of K87E mutation, linked to RP and not to LCA, at the time that emphasize the toxicity of RetGC miss localization to proximal compartments.

Keywords: Photoreceptor cells; Phototransduction; Retinal degeneration 3; Retinal guanylate cyclase; Retinitis pigmentosa.

INTRODUCTION

1. Photoreceptor cells

Light transduction is the first step of the vision process. It takes place at the photoreceptor cells in the back of the retina, where photons of light are captured by the visual pigment rhodopsin (or colour opsins) and converted to

an electrochemical signal that is transmitted to higher order neurons in the retina and ultimately to the brain. Despite its peripheral location, the retina is considered to be a part of the central nervous system (Fain, 2006; Purves and Williams, 2001). Genetic defects that impair the mechanisms of light transduction lead to different types of blindness. A deeper understanding of the mechanisms that underlie the light response in photoreceptor cells of the retina is required in order to design new treatments and therapies for the greatly heterogeneous inherited retinal dystrophies (Rivas and Vecino, 2014; He *et al.*, 2018).

The primary photoreceptor cells in the visual system the rods and cones. Both rods and cones are highly compartmentalized neurons specialized for the capture of light and its conversion to an electrical signal. Rods and cones have a similar structure (Outer segment (OS); Inner segment (IS) and Synaptic ending) (*Fig 1*).

Their most distinguishing feature is the presence of an OS that derives from a primary non-motile cilium that contains a pile of tightly packed membrane discs where the photopigment protein localizes: rhodopsin in rods colour opsins in the three types of cones. A tight packing of the photopigment in the membrane of these discs, where it is organized in an almost crystalline array, sustains the high sensitivity of photoreceptor cells to light that

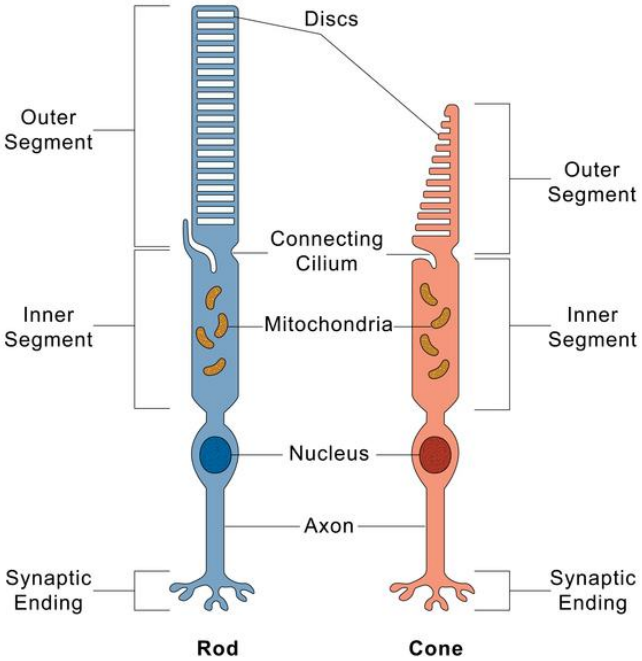

Fig 1: Structural differences between rods and cones. Although generally similar in structure, rods and cones differ in their size and shape, as well as in the arrangement of the membranous disks in their segments (Purves and Williams, 2001).

is, their high capacity to absorb photons (Michael Land, 2018).

The OS, often referred to as "the ciliary compartment" or "sensory antenna" of the cell is where phototransduction takes place. The signalling proteins involved in the light response, besides the photopigment, are organized in the membrane discs or cytosolic space of this compartment.

This compartment is separated from the cell soma by a thin connecting cilium. The cell soma comprises the IS -that contains the endoplasmic reticulum, Golgi apparatus and mitochondria- where most anabolic processes of the cell take place; and the cell nucleus. Finally, an axonic terminal extends that contacts bipolar and horizontal cells (Matte, Laue and Cote, 2012).

Proteins involved in phototransduction are synthesized in the IS of photoreceptor cells and later translocated through the cilium to the outer segment (Azadi, Molday and Molday, 2010).

Despite sharing a common structure, rods and cones also present distinctive features. Rods present a higher density of the photopigment rhodopsin, and have a higher sensitivity to light. Rods are extremely sensitive and are the photoreceptors that sustain vision in low ambient light intensities (black and white scotopic vision). In turn, rods saturate at relatively low light intensities. In contrast cones are less sensitive to light, that is, they have a higher light threshold, but do not electrically saturate and can respond to light at the widely varying, high ambient illumination intensities encountered in the natural world during the day (diurnal, colour, photopic vision)) (Purves and Williams, 2001).

Rods are more abundant in the periphery of the retina and allow for the peripheral vision and detection of contrasts and movement in the vision field, whereas cones are concentrated in the center of the retina including the macula where central visual activity resides. Thanks to their tight packing, cone presents small receptive fields and allow for high-resolution vision. Both types of photoreceptor cells relay on the same signalling module for light transduction: a GTP-binding-protein signalling system.

2. Phototransduction

Phototransduction is the capture of light and its conversion to an electrical signal at photoreceptor outer segments. The absorption of a photon of light by the photopigment (rhodopsin in rods, cone opsins in cones) leads to the photoisomerization of the chromophore, 11-cis-retinal, a covalently attached molecule that derivates from vitamin A. Photoisomerization of the chromophore causes a conformational change in rhodopsin (R) that confers it its active state (R*). During its active lifetime, each rhodopsin molecule activates multiple molecules of the GTP-binding protein transducin (heterotrimer Gα,β,γ), each of which activates a molecule of the effector cGMP-phosphodiesterase (heterodimer αβ of cGMP-PDE). So, light results in an increase in cGMP hydrolysis (Lamb and Pugh, 2006).

The entry of positive charge (Na^+/Ca^{2+} influx) to the outer segments is gated by cGMP (cG), because the Na^+/Ca^{2+} channels respond to the cGMP ligand. The free levels of cGMP set the number of open channels at any illumination condition, and therefore the cationic influx and the membrane potential of the cell (*Fig2* and *Fig3*) (Purves and Williams, 2001; Michael Land, 2018).

In the dark, the cGMP levels are high and the cGMP-gated ion channels are open, so there is an influx of Na^+ and Ca^{2+} ions that has a depolarizing effect on the cell, keeping the membrane potential at approximately -40mV. Because transmitter release at the synaptic terminal is dependent on voltage-sensitive Ca^{2+} channels in the terminal membrane, there is a steady release of glutamate in the dark state.

In response to light, as the cGMP levels drop, the cGMP-gated channels close and there is a reduction in the influx of Na^+ and Ca^{2+}, which has a hyperpolarizing effect on the cell. The effect of light is driving the membrane potential to more negative values (Purves and Williams, 2001). As a result, there is a reduction in the amount of neurotransmitter release at the synaptic terminal.

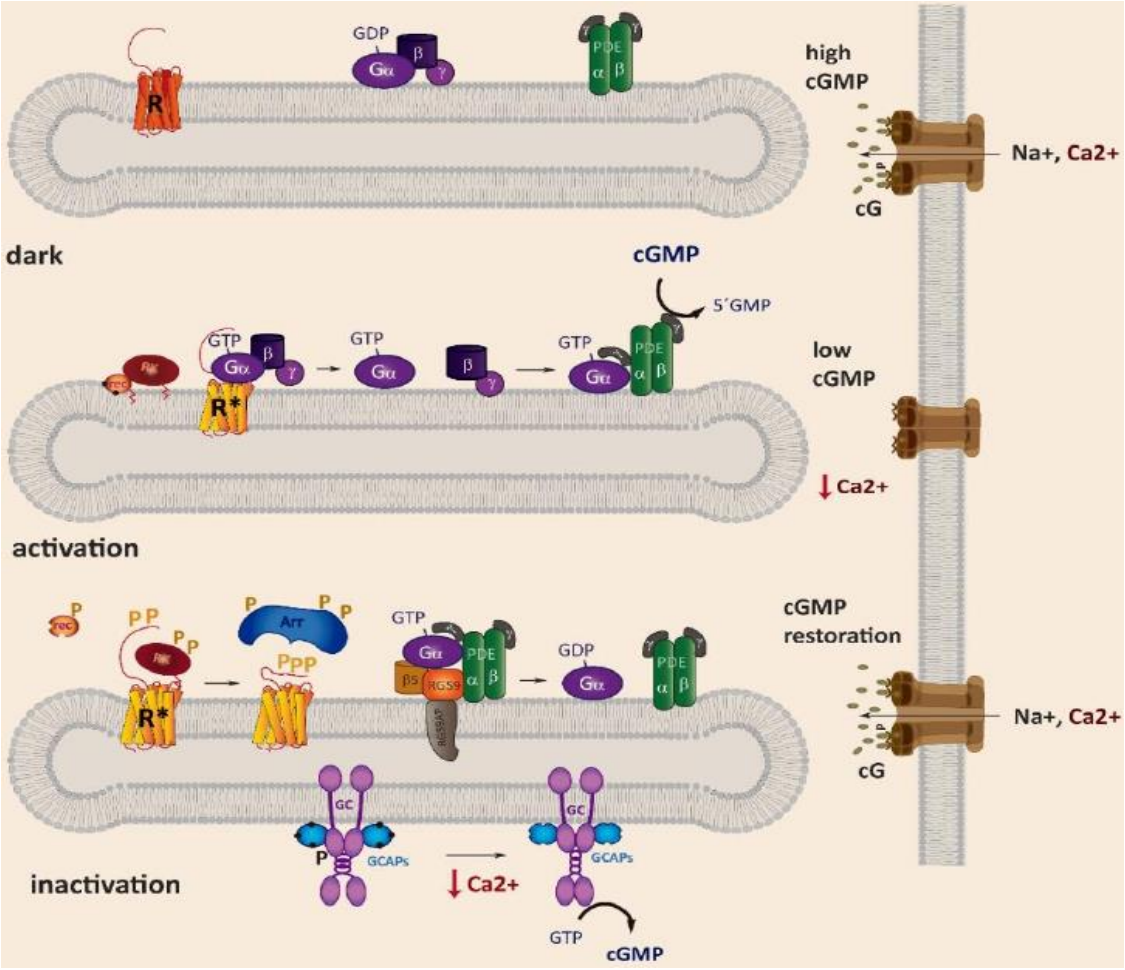

Fig 2. The phototransduction enzymatic cascade at dark, activation and inactivation stages. In a dark stage, there is a hight level of cGMP and cGMP-gated channels are open. The influx of Na^+ and Ca^{2+} has a depolarizing effect on the cell; In the activation stage, the cells consume part of cGMP in response to light. So that cGMP levels drop and the cGMP-gated channels close. There is a hyperpolarizing effect on the cell; In the activation stage, the cells restorage cGMP levels by new synthesis. cGMP-gated channels are open and there is a influx of Na^+ and Ca^{2+} in the cell (Fig made in the lab).

In the recovery phase, the cell regains its darkness equilibrium after the light response. As the light exposure ceases, the photoreceptor terminates the enzymatic response and restores the dark-state. For that, R* and the effector complex (Gα/PDE) are inactivated in a series of inactivation steps; and, in

another essential step, the cGMP levels are restored to the dark levels by new synthesis.

The enzyme responsible for cGMP synthesis is retinal Guanylate Cyclase (RetGC), which is bound to some Ca^{2+}-sensor proteins [Guanylate Cyclase Activating Proteins, GCAPs] that confer it $Ca2^{+}$-sensitivity. As cGMP levels are restored the channels reopen and the inward current is re-established (Purves and Williams, 2001).

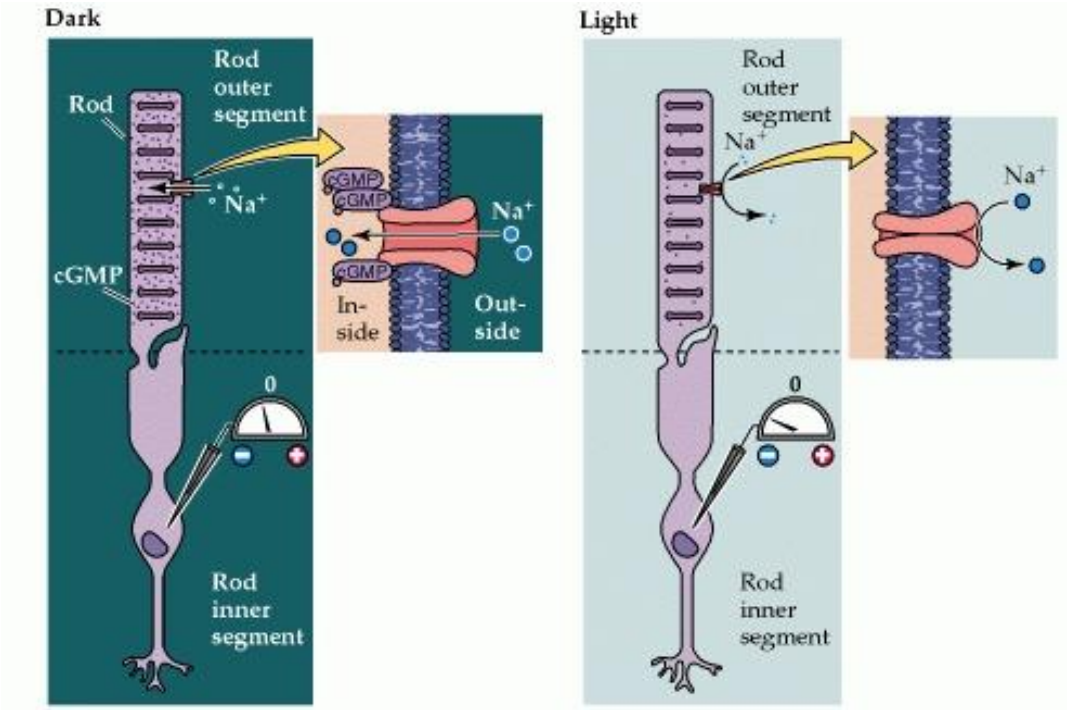

Fig 3. Cyclic GMP-gated channels and light-induced changes in the electrical activity of photoreceptors. This simplified diagram shows a rod, but the same scenario would apply to cones. In the dark, cGMP levels in the outer segment membrane are high; cGMP binds to the Na^{2+}/Ca^{2+} permeable channels in the membrane, keeping them open and allowing sodium and other cations to enter, thus depolarizing the cell. In the light scenario, the absorption of photons leads to a decrease in cGMP levels, closing the cation channels and resulting in receptor hyperpolarization (Purves and Williams, 2001).

3. The importance of RetGC function in termination of the light response

RetGC is a membrane protein that is responsible for the new synthesis of cGMP from GTP. There are two RetGC isoforms, RetGC1 and RetGC2. RetGC1 is found in both cones and rods, and RetGC2 is only found in rods. In humans, RetGC1 is the most abundant isoform and the only one that has been associated to blindness. Therefore, we will here in after refer to both RetGCs as simply RetGC, keeping in mind that RetGC1 is the relevant isoform for humans (Helten and Koch, 2007; Helten, Säftel and Koch, 2007).

RetGC is a transmembrane protein localized at the membrane disks of rods and cones that is active in dimeric form. It has an extracellular domain (ECD) and an intracellular region that contains a Kinase Homology Domain (KHD) and a Catalytic Domain (CD). It is associated by its intracellular region to small soluble calcium-binding proteins from the calmodulin superfamily: the Guanylate Cyclase Activating Proteins (GCAPs). GCAPs act as Ca^{2+} sensors, binding or dissociating Ca^{2+} depending on the intracellular calcium concentration which is set by the illumination conditions**.** **In the dark** when intracellular Ca^{2+} is high (open channels), GCAPs in their Ca^{2+} bound state inhibit cyclase activity. **In response to light**, as the intracellular Ca^{2+} drops due to the closure of the channels, GCAPs dissociate Ca^{2+} and acquire their Ca^{2+}-free activator state. **In their activator state** GCAPs stimulate RetGC activity up to about ten times its basal levels, causing a boost in cGMP synthesis (*Fig4*). This is a key step in termination of the light response and recovery of sensitivity.

RetGC is required for photoreceptor survival. In humans, mutations in GUCY2D gen, that codes for RetGC1, that create premature stop codons yield truncated, inactive forms of the protein have been linked to Leber Congenital Amaurosis 1 (the first described form of LCA), one of the most severe forms of inherited blindness. Although present, RetGC2 cannot compensate for RetGC1 function in humans. In the murine system where RetGC2 is more abundant in rods, RetGC2 can compensate for RetGC1 function in rods (not in cones). A

double knockout in RetGC1/RetGC2 has been developed as a model of LCA1, which reproduces the phenotype of the disease (Boye *et al.*, 2013).

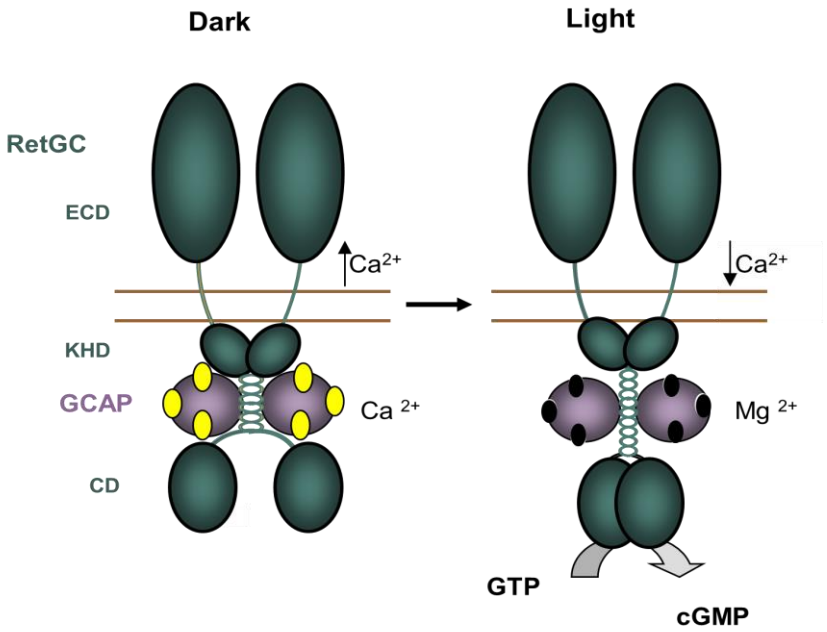

Fig 4. Model of regulation of RetGC by GCAPs. In a dark state Ca^{2+} levels are hight and GCAPs in their Ca^{2+} bound state inhibit cyclase activity. In response to light, intracellular Ca^{2+} drops and GCAPs dissociate Ca^{2+} and acquire their Ca^{2+}-free activator state. In their activator state GCAPs stimulates RetGC activity causing a boost in cGMP synthesis (Fig made in the lab).

4. Retinal Degeneration 3 protein (RD3) has been shown to be required for RetGC trafficking to the outer segment

The mechanisms underlying the transport of signalling membrane proteins from the cell soma (inner segment where proteins are synthesized) to the outer segment where phototransduction takes place has been a focus of attention in the phototransduction field in recent years (Jana Mazelova *et al.*, 2009; Deretic *et al.*, 2005; Karan, Frederick and Baehr, 2008). The reason is that many novel genes that have been recently associated to blindness code for proteins required in the trafficking of important signalling complexes (Nikopoulos *et al.*, 2016; Karlstetter *et al.*, 2014; Di Gioia *et al.*, 2015; Roosing *et al.*, 2014).

Membrane proteins synthesized at the ER must exit the ER to enter the Golgi apparatus, and must cluster at specialized domains at the Trans-Golgi network, where a set of Arf and Rab proteins will attract the proper coat proteins to orchestrate the budding of specialized transport carrier vesicles with ciliary destination. Dedicated "Ciliary Targeting Sequences" in "guide" proteins are required for this process. Examples of two essential proteins in rod outer segments that have Ciliary Targeting Sequences are rhodopsin and peripherin that are known to guide transport carrier vesicles to the cilium (Tam *et al.*, 2000; Deretic *et al.*, 2005; J. Mazelova *et al.*, 2009). The signal sequences in other proteins, if present, are still unknown. This is the case for RetGC. The mechanisms that direct RetGC to the cilium upon synthesis are only beginning to emerge.

RD3 is an evolutionary conserved 23 kDa protein encoded by a locus that has been known to be associated to blindness for a long time. The function of the RD3 protein has been reported more recently (Azadi, Molday and Molday, 2010). The rd3 strain of mice is one of the oldest natural strains identified at Jackson´s Laboratories (Maine, USA) that present retinal degeneration and blindness. It is caused by a mutation in the called rd3 locus (from retinal degeneration 3), that introduces a premature stop codon after residue 106, leading to a truncated form of the protein that is rapidly degraded. In humans, a truncation in the rd3 locus causes the same phenotype that null mutations in GUCY2D: Leber Congenital Amaurosis. As rd3 was the twelfth locus associated to LCA, it is said that a mutation in RD3 causes LCA12.

A few years ago Dr. Robert Molday´s group in Canada generated specific antibodies to the 23 kDa expressed RD3 protein and performed immunoprecipitation assays in whole murine retinas. His group found that RD3 binds to RetGC, and is absolutely required for RetGC stability and trafficking *in vivo*. The rd3 mice had largely reduced levels of RetGC; and somewhat reduced levels of the GCAP proteins, that were retained at proximal compartments and failed to be distributed to the outer segment compartment.

The authors proposed that RD3 was specifically required to extract RetGC from the endoplasmic reticulum, as in the absence of functional RD3 in rd3 mice traces of RetGC were observed in the ER (Azadi, Molday and Molday, 2010).

Furthermore, the reintroduction of the rd3 gene in the rd3 mice by using adeno-associated virus 8 (AAV) restored the expression of RetGC (RetGC1 and RetGC2) and its localization to rod and cone outer segments. Thus, RD3 was proposed to be required for the stability and trafficking of RetGC (Azadi, Molday and Molday, 2010).

The laboratory of Dr. Alexander Dizhoor (Pennsylvania, USA) subsequently investigated the effect of RD3 on RetGC catalytic activity. Peshenko et al. showed that RD3 acted as a non-competitive high-affinity inhibitor of RetGC, abrogating RetGC function even in the presence of GCAPs. RD3 did not compete with the substrate but competed with GCAP1, precluding GCAP1 activation of the cyclase. Peshenko et al. therefore proposed that RD3 is a potent RetGC inhibitor that prevents cGMP synthesis while RetGC is "in transit" to the outer segment (Peshenko *et al.*, 2011a).

According to Peshenko and Dizhoor´s model, RD3 is excluded from the outer segment compartment. This is in direct conflict with Molday´s original study, which reported RD3 main localization at the rod outer segment layer. Furthermore, the immunoprecipitation assays that led to RetGC identification as RD3 targets were performed using murine outer segment preparations.

In brief, there is controversy over RD3 localization in vivo. The antibodies used for RD3 initial studies have been questioned by other groups (Koch and Dell'Orco, 2015).

Here in our lab we are interested at studying the mechanisms underlying the trafficking of RetGC/GCAP complexes. Therefore the lab is interested at understanding RD3 role in this process, and in solving the RD3 localization controversy. The lab has developed a procedure for the transfection of tagged-RD3 expression vectors in photoreceptor cells of the retina in living mice, by *in*

vivo DNA electroporation of mice after plasmid DNA injection in the subretinal space. By expressing the RD3 protein fused to a tag, the lab is immunolocalizing the protein by using establishes antibodies against the tag (V5 epitope).

Because there is a single mutation in RD3 that has been linked to autosomal recessive retinitis pigmentosa (arRP), that results in the K87E substitution, my project was set to study whether this substitution alters RD3 (and RetGC) subcellular localization *in vivo.*

5. Retinal distrophies

Retinal distrophies (RD) are defects in the cells of the retina that lead to vision impairment. Retinal dysfunctions are common causes of blindness. Mutations in genes involved in light response or dark adaptation lead to malfunctioning cells that eventually die (De Castro-Miró *et al.*, 2014; Fain, 2006).

There are more than 25 genetic visual disorders and 5000 mutations in almost 200 genes involved in RD. However, the most frequent form of RD is retinitits pigmentosa (RP) that affects 1.5 milions individual woldwild (De Castro-Miró *et al.*, 2014). This diversity of genetic pathophysiology makes treatment extremely challenging.

According to the genetic defect either the affected cells, RD can be classified as: rod-dominant abnormality, cone-dominant abnormality, macular dystrophy, abnormality of photoreceptors and bipolar cells, vitreo retinopathies and hereditary choroidal diseases. The most frequent and severe forms of retinopathies are the rod-dominant abnormalities or RPs (Dias *et al.*, 2018).

The first sign of RP is usually a loss of night vision, which becomes apparent in childhood. Problems with night vision can make it difficult to navigate in low light. Mutations in more than 60 genes are known to cause RP. Those genes play essential roles in the structure and function of rods (Daiger *et al.*, 2008).

Mutations in any of the genes responsible for RP lead to a gradual loss of rods and subsequently cones in the retina. The progressive degeneration of these cells causes the characteristic pattern of vision loss. Rods typically break down before cones, which is why night vision impairment is usually the first sign of the disorder. Daytime vision is disrupted later, as both rods and cones are lost. A lot of gene mutations have been described to cause RP (Daiger *et al.*, 2008;Dias *et al.*, 2018).

Up to now, mutations in rd3 gen was only associated to Leber Congenital Amaurosis. Recent studies discovered a mutation (K87E) in rd3 gen that was identified as causative of RP, by using novel genetic diagnostic tools that use available genetic information to prioritize candidates for mutational screening, and then use massive next generation sequencing (De Castro-Miró *et al.*, 2014).

In this study we want to study the mechanisms governing Rd3 subcellular distribution in photoreceptor cells by studying the molecular determinants of distribution *in vivo*. Likewise, we want to compare these results to K87E Rd3 mutation subcellular distribution *in vivo*.v

AIMS

The main aim of this project is to gain inside into RD3 function in photoreceptor cells *in vivo* by studying the mechanisms underlying the trafficking of RetGC/GCAP complexes. Therefore the lab is interested in understanding RD3 role in this process, and in solving the RD3 localization controversy.

Furthermore there is a single mutation in RD3 that has been linked to autosomal recessive retinitis pigmentosa (arRP), the K87E substitution, my project was set to study whether this substitution alters RD3 (and RetGC) subcellular localization *in vivo*.

First aim

Analyze RD3 subcellular localization by using a genetic approach, thereby circumventing the use of anti-RD3 antibodies.

Second aim

Study whether the blindness causing mutation K87E affects RD3 subcellular localization and/or guanylate cyclase distribution to the outer segment compartment.

MATERIALS AND METHODS

1. Generation of the expression vector pRho_K87E/mRd3V5_dsRed

Mutation K87E was introduced in the expression vector pRho_mRd3V5_dsRed, by using the QuickChange Lightning Site-Directed Mutagenesis 2961Kit *(*Agilent Technologies*).*

pRho_mRd3V5_dsRed (*Fig 5*) is a bicistronic expression vector that was designed to express the mouse RD3 cDNA (dark green) with a V5 tag at the COOH-terminus (purple) under the control of the bovine rod opsin promoter (green). This promoter also controls the fluorescent marker dsRed (Red) that is localized after an Internal Ribosome Entry Site (IRES) sequence (blue). Moreover the plasmid also contains the bacterial SV40 replication origin (SV40ori) (yellow) and an ampicillin resistance gene (light green) controlled by the ampicillin promoter (dark blue) to perform the selection in bacteria.

For site-directed mutagenesis, the QuickChange Lightning Site-Directed Mutagenesis 2961Kit (Agilent Technologies, USA and Canada) was used according to manufactures´ instructions. Basically, the procedure consisted on amplifying the pRho_mRd3V5_dsRed vector (in super coile double-stranded form) with two synthetic oligonucleotide primers, both containing the desired

mutation shown in red. The sequence of the forward primer (Fw_K87E_mRD3) was GGAGGATGTATGTGCCGAGATCCACCCATCCTA and that of the reverse primer (Rv_K87E_mRD3) was TAGGATGGGTGGATCTCGGCACATA CATCCTCC.

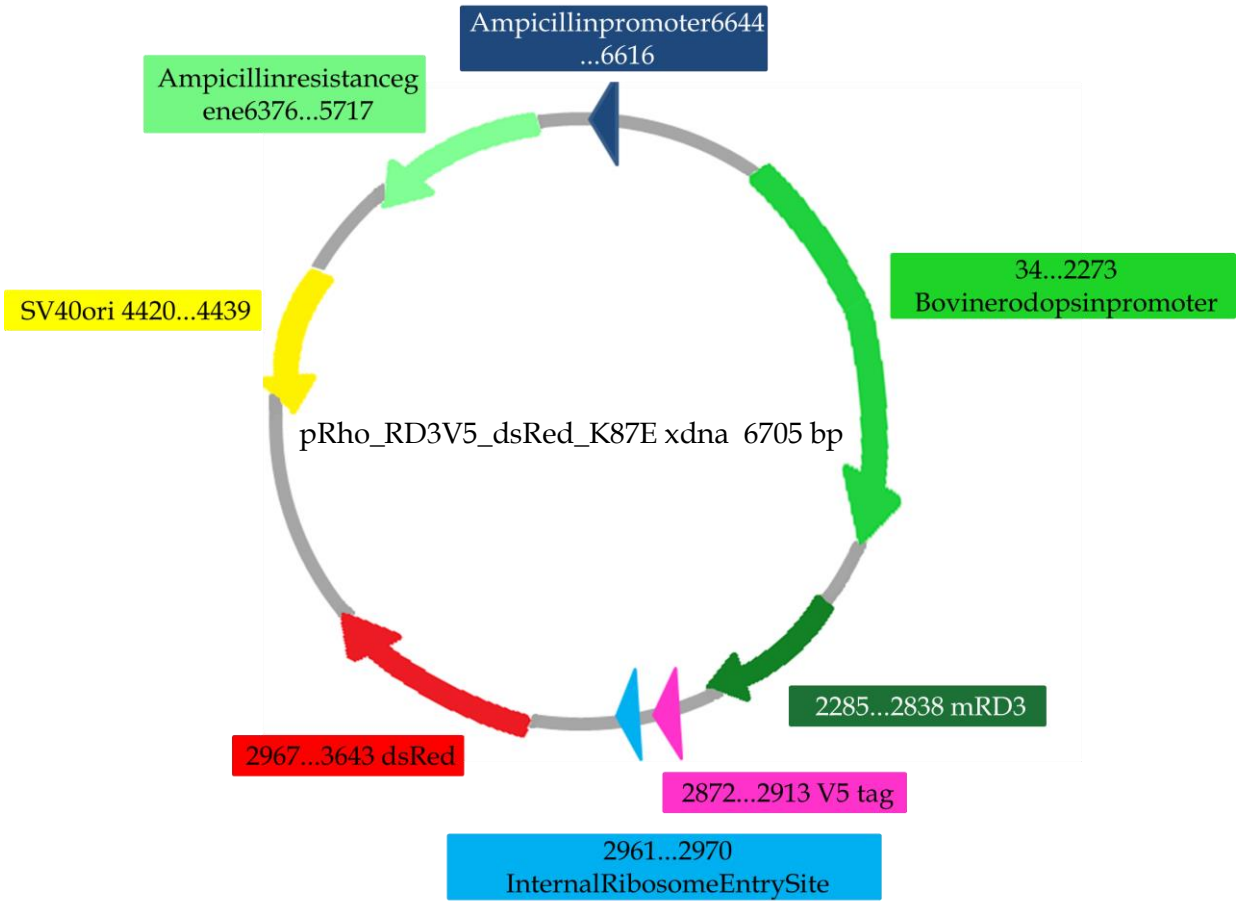

Fig 5. pRho_K87E/mRd3V5_dsRed plasmid

Primers were designed with a melting temperature over 80ºC, trying to avoid primer dimerization, by using the Sygma Aldrich program. The alignment between primers and template vector was checked by using Serial Cloner program.

Polymerase Chain Reaction (PCR) amplification was performed with the PfuUltraHF DNA polymerase (indicated to amplify large fragments of DNA rapidly, with proof reading activity). Eighteen cycles were performed at an

annealing temperature of 60ºC, using 3,5 min in the elongation step (My Cycler™ Thermal Cycler System BIO-RAD, USA) (*Table1*).

Table 1. Cycling parameters for the QuickChange Lightning Site-Directed Mutagenesis Method (Agilent Technologies).

Segment	Cycles	Temperature	Time
1	1	95ºC	2 minutes
2	18	95ºC	20 seconds
		60ºC	10 seconds
		68ºC	3,5 minutes
3	1	68ºC	5 minutes

Following the PCR cycling, the product was treated with 2 µL of *Dpn I* (10U/µL) for 1hour at 37ºC. The *DpnI* endonuclease is specific for methylated and hemymetilated DNA and is used to digest the parental DNA template.

Finally XL10-Gold Ultracompetent Cells were used for transformation by thermal shock, because they have the capacity to repair DNA nicks. Transformed cells were seeded in LB/Amp agar plates and grown at 37ºC overnight. A schematic procedure of the QuikChange Lightning site-directed mutagenesis method was shown in (*Fig6)*.

Plasmid DNA from transfected bacterial colonies was amplified and purified by miniprep (NucleoSpin®Plasmid. MACHEREY-NAGEL) and quantified by using an IMPLEN nanophotomer (bioNova cientifica, s.l). To check the purified plasmid, an enzyme digestion was performed with AseI and BbsI restriction enzymes.

The plasmid was sequenced to confirm the desired mutation was introduced.

Once we confirmed that the plasmid pRho_K87E/mRd3V5_dsRed had been properly generated, it was amplified and purified for *in vivo* DNA injection, by using the PureLinkTM Expi Endotoxin-Free Mega Plasmid Purification Kit (Invitrogen, Thermo Fisher Scientific) and quantified by using an IMPLEN nanophotomer (bioNova cientifica, s.l).

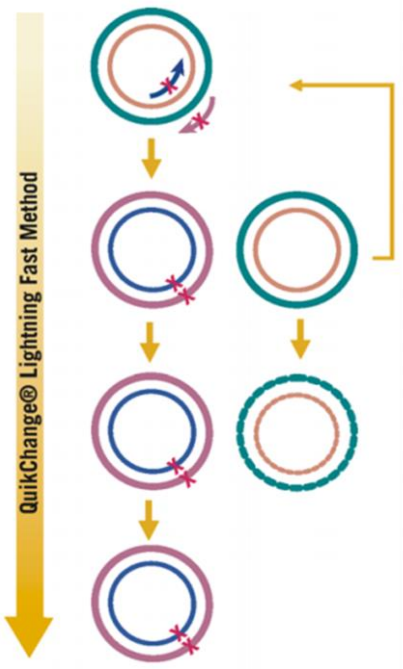

Fig 6. schematic procedure of the QuikChange Lightning site-directed mutagenesis method. A) Mutant strand synthesis by using thermal cycling. First of all there is a DNA template denatures. Secondly there is a mutagenic primers containing desired mutation annealing. Finally, extend and incorporate primers with *Pfu*-based DNA polymerase Blend; B) Faster *Dpn* I digestion of template stand. Digest parental methylated and hemimetilated DNA; C) Transformation. The plasmid are ready be transformed into competent cells for nick repair.

2. Generation of transgenic mice by transient gene expression in the retina: *In Vivo* DNA injection and electroporation

The mice used in our research were treated according to Acts 5/1995 and 214/1997 for the welfare of experimental animals of the Autonomous Community (Generalitat) of Catalonia, and approved by the Ethics Committee on Animal Experiments of the University of Barcelona.

In vivo DNA electroporation in the retina following circular DNA injection in the subretinal space is a technique that allows transient expression of a gene of interest into photoreceptor cells, when a photoreceptor-specific promoter is used. Here we followed the procedure developed by C. Cepko's laboratory at Harvard in 2004 (Matsuda and Cepko, 2004).

Despite our expression vector were designed to have bicistronic expression of dsRed, we found that the expression of dsRed after an IRES sequence is very low. For that reason, in order to easily identify the injection area during the injection procedure, a DNA solution in PBS and fast green 0,1% dye was prepared by mixing the pRho_mRd3V5_dsRed plasmid (or

pRho_K87E/mRd3V5_dsRed) with a dsRed encoding plasmid at a molar ratio of 4:1.

In order to analyze RD3 subcellular localization, as well as K87E/RD3 subcellular localization, the corresponding expression vectors were injected and electroporated in rd3/rd3 mice, lacking functional endogenous RD3, and in GCAPs -/- mice the day they were born (newborn pups).

Briefly, the procedure involved anesthetizing the newborn pups by chilling them for four minutes submerged on ice. Once anesthetized, the right eye of each mouse was electroporated. So that, a small incision was made following the natural eyelid line by using a sterile razor blade, so that the eyelid was opened. Then one hole was made in the cornea with a 30-gauge needle and 0.2-0.5 µl of DNA was injected into the subretinal space with a Hamilton syringe with a blunt 30-gauge needle undera dissecting microscope (Zeiss KL1500$_{LCD}$. Stemi2000, Germany). After DNA injection, tweezer-type electrodes briefly soaked in PBS 1X were placed to softly hold the head of the pup, and five 80V square pulses of 50ms duration with 950ms intervals were applied by using a CUY21 electroporator (Nepagene, Chiba, Japan).

At the end of procedure, in order to prevent infections the pup injected eye was treated with povidone iodine 10% and pups were returned to their cage and raised in normal conditions. Mice were processed for immunological staining at postnatal day 20.

3. RD3 subcellular localization studies by immunofluorescence analysis in retinal sections

To obtain retinal sections for immunofluorescence analysis, mice were sacrificed and retinas were processed implemented the procedure previously described with minor modifications (Hoyo *et al.*, 2014; López-del Hoyo *et al.*, 2012).

Immediately after enucleation the eyes were punctured with a needle and submerged in fixative (4% paraformaldehyde in 1xPBS, pH7.4). At 5 min into the fixation step, the cornea was excised; and at 15 min the lens was removed and the eye cups were further fixed for a total of 1 hour and 15 minutes at room temperature. Eye cups were infiltrated in acrylamide over night before acrylamide polymerization was induced. Specimens were included in OCT compound and frozen in liquid nitrogen. Cryosections along the vertical axis of the eyecup were obtained at 20 µm-thickness using a CM1510S Leica cryostat (Leica Microsystems). After that, cryosections that covered the injected areas of the retina were selected by looking for sections that showed red fluorescence due to dsRed reporter expression (Nikon eclipse e800 JENOPTIK, Germany). Those sections were frozen at -80^{o}C until used.

The subcellular immunolocalization of RD3 and RetGC was assayed in transfected retinas with the following antibodies:

- Anti-V5 mAb [(2F11F7) 5mg/ml Invitrogen, 1:200 made in mouse]
- Anti-bRetGC pAb (custom Ab made in rabbit, against the COOH-terminus of the protein 1:200)

Secondary antibodies were α-mouse Alexa Fluor 647 (LifeTechnologies; Carlsbad, CA, USA) and α-rabbit Alexa Fluor 488 (LifeTechnologies; Carlsbad, CA, USA 1:500).

The following protocol was used: cryosections underwent an antigen retrieval protocol prior to the blocking step (proteinase K 0.05mg/ml, 2min room temperature, and 70^{o}C 8sec). Primary antibody incubation was performed for 16h at 4^{o}C; secondary antibody incubation was performed for 1h at room temperature.

After the incubations, sections were fixed for 15min in 4% paraformaldehyde and mounted with Mowiol (Calbiochem, San Diego, CA, USA).

Images were obtained at a laser scanning confocal microscope (Zeiss LMS880) by using the objective plan-apochromat 63x/1.4 oil

RESULTS

To assess the subcellular distribution of RD3 in photoreceptor cells, rd3 mice were electroporated with plasmid pRho-RD3.V5-dsRed after *in vivo* DNA subretinal injection. This allowed the transgenic expression of RD3.V5 in transfected photoreceptor cells, resulting in retinas showing the mosaic expression that characterizes retinal DNA electroporation experiments (*Fig 7*).

1. RD3.V5 localization in rd3 mice

RD3.V5 localization in rd3 mice was observed at the cytosolic space of proximal compartments of rod photoreceptor cells: inner segment, perinuclear region and synaptic terminal, being most prominent at the inner segment compartment (*Fig 7* upper panels, magenta signal). RD3 also localized at the base of the outer segment compartment, forming a characteristic "staple-like" structure. However, RD3 signal was not observed throughout the length of the outer segment (ciliary compartment). Importantly, transgenic expression of RD3.V5 restored the expression and normal distribution of RetGC1 in transfected rod photoreceptors (green signal at the rod outer segments, observed only in transfected rod cells, *Fig 7* upper panel). Non-transfected cells did not show RetGC1 signal at the outer segment compartment, due to the lack of functional RD3 in the rd3 mouse model. **This result shows that transgenic RD5.V5 is fully functional *in vivo* at restoring normal RetGC1 expression and trafficking to the outer segment and that the RD3 protein main localization is the inner segment compartment.** In *Fig 7*, the red channel shows dsRed expression in the nuclei of transfected cells. Note that due to the 4:1 molar ratio

of transfected plasmids pRho-RD3.V5-dsRed and pRho-dsRed, nearly all transfected cells express Rd3.V5, but not all transfected cells express dsRed.
Because RD3 and the GCAP proteins compete for their binding to the cyclase, which is mutually exclusive (Peshenko *et al.*, 2011a), we reasoned that RD3 localization could be affected in the absence of GCAP proteins. Therefore, we assessed RD3 localization in the GCAPs-/- mouse model, lacking both GCAP1 and GCAP2 (Mendez *et al.*, 2001).

2. RD3.V5 localization in GCAPs-/- mice

RD3.V5 localization in GCAPs-/- mice showed that RD3.V5 distribution was altered in the absence of GCAPs. In GCAPs-/- mice, RD3.V5 distributed to the outer segment compartments of transfected cells (*fig 7* white arrows, magenta channel of middle panel). Note that this is observed in photoreceptor cells that were transfected with pRho-RD3.V5-dsRed only. Those photoreceptor cells that acquired both plasmids and express dsRed at high levels (strong red nuclei in red panel) are visually misleading, due to cross-talk of the red signal at rod inner segments in the magenta channel. For that reason, we scanned injected eyes for areas with low levels of transfection, and selected cells with low dsRed expression (*fig 7* lower panels). In transfected cells with low dsRed expression – no cross talk between red and magenta channels- it was evident that RD3.V5 distributed to the ciliary (rod outer segment) compartments in GCAPs-/- rods. GCAP-/- mice show normal RetGC1 expression and distribution (green channel). **This result shows that RD3.V5, in the absence of GCAPs, distributes to the outer segment compartment**. Therefore we infer that in the normal physiological scenario –presence of GCAPs-, the GCAP proteins compete with RD3 for binding to RetGC1 at some point in the trafficking pathway to the cilium (very likely at connecting cilium or the base of the outer segment), displacing RD3 from the cyclase. RD3 would therefore be "recycled" to the inner segment, without being distributed to the outer segment. We envision a sequential mechanism of RetGC1 trafficking, in which RD3 is

required for the trafficking of RetGC1 to the base of the cilium in a first step, that would be followed by a second step in which GCAPs displace RD3 and form a stable complex with RetGC1 at the base of the outer segment, so that RetGC at the outer segment compartment is all bound to the GCAP proteins (see Discussion).

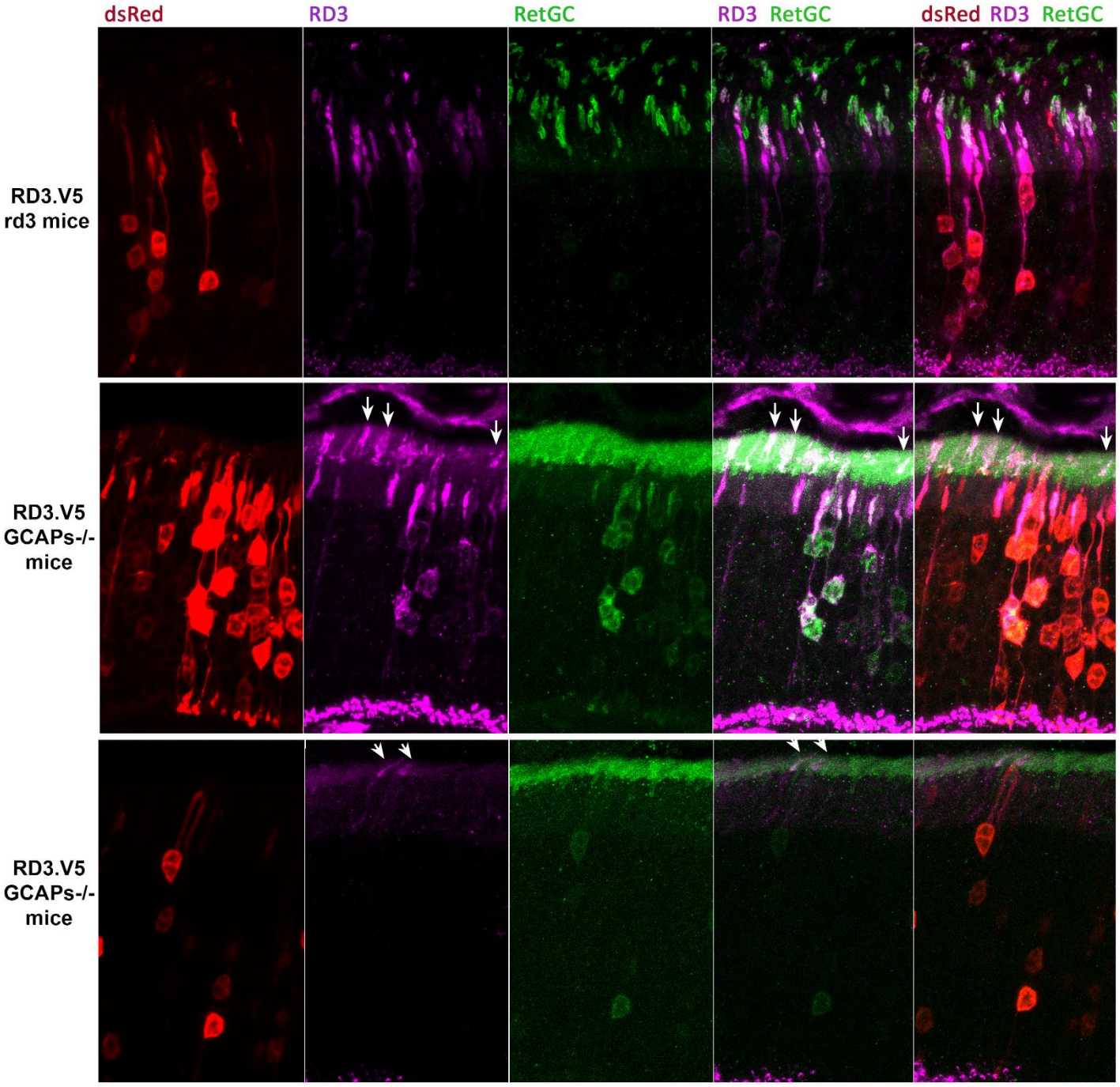

Fig 7. Subcellular localization of RD3 in photoreceptor cells of rd3 and GCAPs-/- mice. The RD3.V5 fusion protein expressed from pRho_RD3.V5_dsRed plasmid in

transfected photoreceptor cells was stained with an anti-V5 antibody (647 channel in magenta). RetGC1 was immunostained with an anti-RetGC1 antibody and visualized with an anti-rabbit IgG Ab (488 channel in green). The dsRed reporter (expressed from pRho_dsRed plasmid) used to mark the injected area of the eye is observed in the 555 channel in red. In rd3 mice, RD3 localizes mostly at the inner segment, although it is also observed at the perinuclear region and cell processes. In rd3 mice, RD3.V5 transgenic expression restores the expression and normal distribution of RetGC1. In GCAPs-/- mice, RD3 distributes to the outer segment compartment of transfected cells.

3. The effect of blindness-associated K87E RD3mutation

The effect of blindness-associated K87E RD3mutation in protein localization and function was studied by transfecting pRho-K87E/RD3.V5-dsRed in rd3/GCAPs-/- mice. The use of rd3/GCAPs-/- mice was circumstantial, due to higher –fortuitous- availability of rd3/GCAPs-/- than rd3 mice for electroporation experiments. Electroporation of the control protein RD3.V5 in rd3/GCAPs-/- mice showed the expected localization (inner/outer segment distribution, magenta signal upper panel Fig 8). It restored RetGC1 expression to the outer segment compartment of electroporated cells. The mutant protein K87E/RD3.V5 showed a similar distribution (lower panel). However, while the control protein RD3.V5 sustained normal RetGC distribution to the outer segments (upper green panel), the mutant K87E/RD3.V5 sustained it only partially, resulting in an increased punctated staining of RetGC1 at proximal compartments of the cell (lower green panel). This result indicates that K87E mutation is likely to decrease the affinity of RD3 for the cyclase, resulting in a partially impaired protein in vivo. However, K87E mutation did not show a complete loss of function, like the truncated form of RD3 expressed in rd3 mice (Azadi, Molday and Molday, 2010; Dizhoor, Olshevskaya and Peshenko, 2019).

We suggest that this partial retention of function of the K87E/RD3 mutant is what explains its association to retinitis pigmentosa (mild phenotype), rather

than Lebers Congenital Amaurosis (LCA, nearly complete blindness from a very early age).

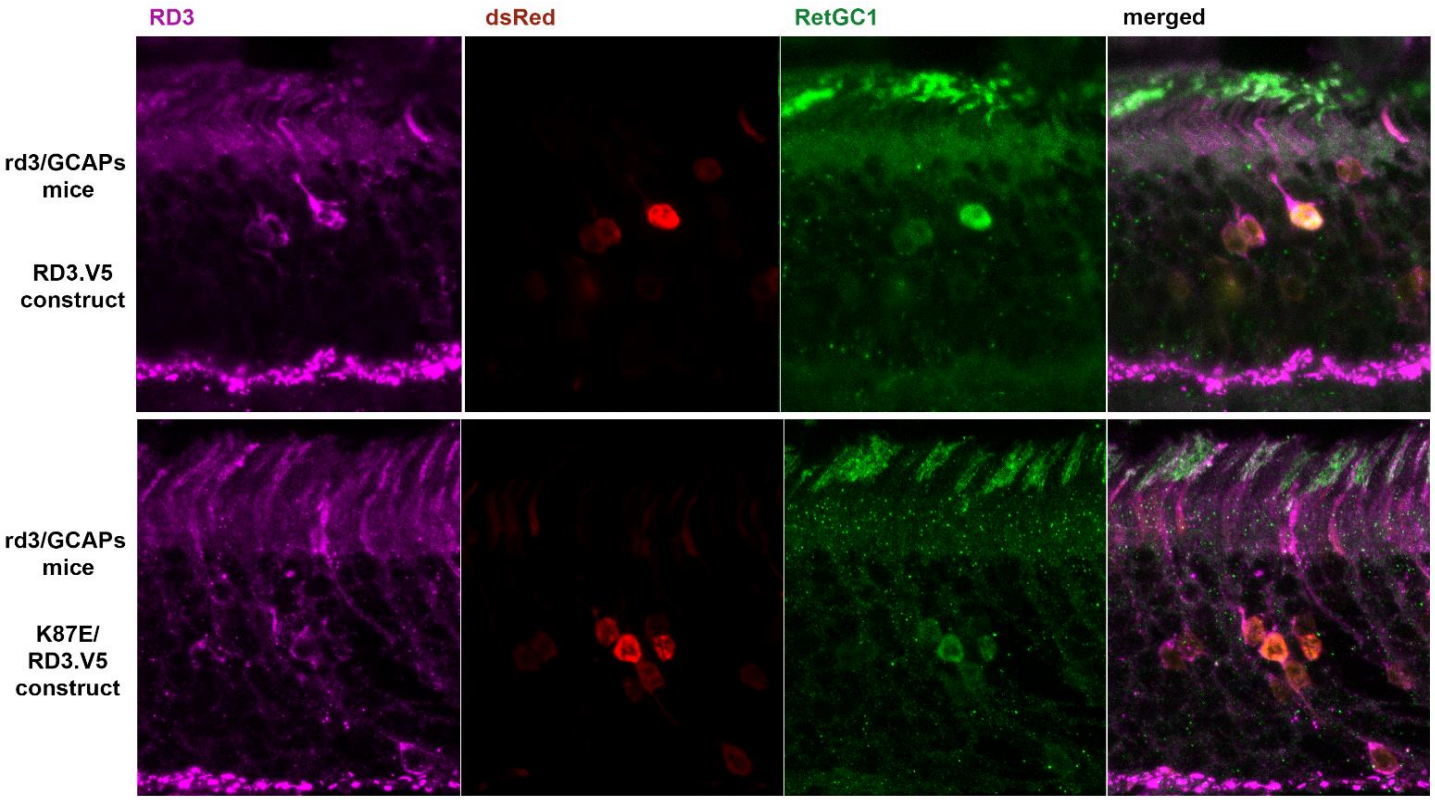

Fig 8. K87E mutation in RD3 reduces RD3 capacity to stabilize and transport RetGC1. In K87E/RD3 transfected photoreceptor cells (from rd3/GCAPs-/- mice), RD3 distributes between the inner and outer segments (as expected in the GCAPs-/- background), and retains the ability to stabilize RetGC1 and facilitate its transport to the outer segment compartment. However, a fraction of RetGC1 is misslocalized to proximal compartments (note the substantially increased punctated green staining in the green channel, in K87E/RD3 cells (lower panel) versus wild type (upper panel).

DISCUSSION

The subcellular localization of RD3 has been an unresolved issue, due to contradicting reports from different studies. The original study that identified RD3 as a RetGC binding protein that promoted its stabilization and trafficking (Azadi, Molday and Molday, 2010) reported RD3 localization at the outer

segment compartment. In contrast, the study that identified RD3 as an inhibitory protein of RetGC activity with very high affinity *in vitro* (Peshenko *et al.*, 2011b) postulated that RD3 should be retained at the inner segments and excluded from the outer segment. Peshenko et al. reasoned that RD3 could not localize to rod outer segment compartments given that it would silence the cyclase at the compartment where the cyclase should exert its role.

Contradictory results are not infrequent in immunolocalization studies of a novel protein, and typically result from inespecificity of generated antibodies (that might recognize the protein of interest and other proteins), or from epitope masking effects of the protein of interest in a particular compartment. RD3 is not a very abundant protein and it is strongly associated to the membrane, being somehow technically difficult to detect (routinely in our lab). For that reason we here circumvented the use of anti-RD3 antibodies, and used a genetic approach to assess RD3 subcellular localization in rod photoreceptors. By expressing a fusion protein of RD3 with a well established tag (V5) in living rods by transient transgenesis, we could then use anti-V5 antibodies to establish RD3 localization. We determined that RD3 localization was most prominent at the inner segment compartment, when RD3.V5 was expressed in the rods of rd3/rd3 mice. This result basically backs Pesenko´s postulate that RD3 should be mostly retained at the inner segment. Actually, while we were carrying out this work, a publication came out from the laboratory of Alexander Dizhoor that analyzed RD3 localization by expressing RD3.GFP in the rods of rd3/rd3 mice by stable transgenesis. Their transgenic line shows RD3.GFP retention at the rod inner segment compartment (Dizhoor, Olshevskaya and Peshenko, 2019). One subtle but putatively important difference, though, was that we also identified RD3 at a structure that resembles the connecting cilium, where RD3 and RetGC1 signals overlap (*Fig 7*).

We have also found that in the GCAPs-/- the RD3 signal distributes to the whole length of the outer segment, which indicates that in the physiological scenario the GCAP proteins are required to displace RD3 at the base of the

cilium. This result was consistent with Dizhoor recent observation that RD3.GFP also distributes to the outer segment in the absence of GCAPs (Dizhoor, Olshevskaya and Peshenko, 2019). We envision a RetGC1 trafficking mechanism consisting of two steps: a first step in which RD3 stabilizes RetGC and escorts it to the base of the cilium while inhibiting its enzymatic activity and a second step in which the GCAPs come into play, binding to the cyclase and displacing RD3 that returns to the inner segment. This "relay step" likely takes places at a very specific location of the cell (the connecting cilium), and likely involves a particular enzymatic step like the phosphorylation of RD3. In any event, RetGC would enter the outer segment bound to the GCAP proteins. Figuring out the missing details of this mechanism will be the next goal of the lab.

The rd3 mouse model expresses a truncated form of the protein that ends at aa105. This truncated form of the protein is unstable and is degraded *in vivo*, so that the rd3 mouse is a model of loss-of-function. Most characterized human mutations in RD3 are equivalent to the truncating mutation in the rd3 mouse, and lead to a very severe form of inherited blindness: Lebers Congenital Amaurosis, LCA12. However, one mutation was found by the group of Roser González Duarte at the University of Barcelona, the K87E mutation, that was clinically linked to retinitis pigmentosa (a milder phenotype that results primarilyin night blindness by compromised rod function, and only later in life in the failure of cones and total blindness) (De Castro-Miró *et al.*, 2014). We here analyzed the effect of the K87E mutation on RD3 localization and RetGC distribution. We here show that K87E/RD3 only partially sustained RetGC distribution to the rod outer segment, with RetGC being also present at the proximal compartments of transfected rods, that showed the punctated staining typical of rd3/rd3 photoreceptors. It has just been proposed that RetGC activity at the inner segment of photoreceptor cells would be the leading cause of cell death in the rd3 mice (Dizhoor, Olshevskaya and Peshenko, 2019). Therefore, we conclude that the milder phenotype associated to K87E mutation

results from its partial retention of function. Its pathology, on the other hand, results from RetGC partial localization at proximal compartments, where it results in toxicity and cell death. Future experiments will be addressed at figuring out the mechanisms by which retGC activity is toxic at the inner segment compartment.

CONCLUSIONS

1. RD3 localizes at proximal compartments of rod photoreceptor cells, being most abundant at the inner segment compartment. However, it is also present at the connecting cilium, where it co-localizes with the cyclase. It is mostly excluded from the whole length of the outer segment.

2. K87E RD3 mutation results in a partial loss-of-function in vivo, by failing to sustain RetGC distribution to the outer segments completely. RetGC misslocalization to proximal compartments is observed in cells expressing K87E/RD3, that likely constitutes the basis of the pathology in the associated form of RP.

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SELF-ASSESSMENT

This project has been performed during my internship in Dr. Ana Méndez Zunzunegui's lab. This opportunity has allowed me to know better the world of basic research into a much deeper level and it has also been a very important step in my way to the professional world.

Working on this project has been a challenge for me. My first months of the internship were very complicated because I had never studied the anatomy of the eye during my university studies. Understand all the proteins involved in phototransduction cascade or the entire molecular scenario in the vision procedure, takes me a lot of time and hours of reading scientific articles.

For that reason, I have been able to understand the amount of people and years of work that each article or scientific discovery represents. Moreover that, allow me to understand the scientific literature from a more professional point of view.

In general the evaluation of the work I have done was positive. This internship has allowed me to get the capability to develop my critical capacity at the same time that it helps me to refresh and understand clearer concepts and laboratory techniques learned during my university studies.

ANNEX I. TUTOR TRACKING SHEET

Normativa de Treball Fi de Grau Facultat d'Enologia
Aprovada per Junta de Facultat d'Enologia del dia 30 d'octubre de 2014

ANNEX 2

FITXA DE SEGUIMENT DEL TUTOR/A del TFG

Nom i Cognoms de l'Alumne/a: ALBA SOLER BORONAT

Nom i Cognoms del Tutor/a: JAVIER UGARTE CHICOTE

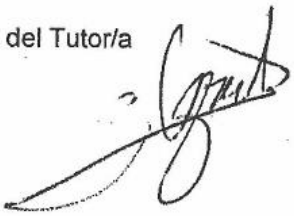
Data de la entrevista amb l'alumne: VARIOS CONTACTOS A TRAVÉS DE CORREO ELECTRÓNICO DADO QUE LAS PRÁCTICAS SON REALIZADAS EN OTRA CIUDAD. ÚLTIMA VERSIÓN ENVIADA 18/07/2019

Recomanacions durant el seguiment:LA INFORMACIÓN QUE SEA PLASMADA EN EL TRABAJO DE FIN DE GRADO DEBE ESTAR REDACTADA Y EXPLICADA DE TAL MANERA QUE PUEDA SER ENTENDIDA POR GENTE AFIN AL TEMA DE TRABAJO PERO NO SER ESPECIALISTAS EN EL TEMA. USO DE ARTÍCULOS PUBLICADOS CON LA TEMÁTICA PARA PODER TENER UNA REFERENCIA DE COMO PUEDE SER PLASMADA LA INFORMACIÓN DE MANERA CLARA Y COMPLETA.

Observacions: LA ALUMNA SE PREOCUPA POR PONERSE EN CONTACTO PARA QUE PUEDA SER REVISADO SU TRABAJO. HA MOSTRADO EL ESFUERZO DE REDACTARLO EN INGLÉS. LA PARTE DE INTRODUCCIÓN FUE ENTREGADA BASTANTE PRONTO DE TAL MANERA QUE SE PUDO OBSERVAR QUE LA ALUMNA SE HABÍA PUESTO AL DÍA EN LA TEMÁTICA BASTANTE TEMPRANO DENTRO DE SU PERIODO DE PRÁCTICAS.

Observacions Darrera revisió:
LA PARTE DE INTRODUCCIÓN DEL TFG SE PODRÍA DAR PRÁCTICAMENTE POR CERRADA. POR OTRO LADO LA PARTE DE METODOLOGÍA HA SIDO CONSIDERABLEMENTE COMPLETADA Y A FALTA DE CAMBIOS MÍNIMOS PODRÍA DARSE TOTALMENTE POR ZANJADA. DEBIDO A CONTRATIEMPOS EXPERIMENTALES SE HA PRODUCIDO UN RETRASO EN LA OBTENCIÓN DE RESULTADOS Y POR LO TANTO EL RESTO DE APARTADOS NO ESTÁN COMPLETOS.

Signatura del Tutor/a



Signatura del Alumne/a

Tarragona a 25 de JULIO 2019