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"Mutation-independent treatment of autosomal dominant *Retinitis Pigmentosa* (adRP)"

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LIST OF ABBREVIATIONS

ZFP, zinc finger protein

ZFRs, zinc finger-based repressors

hRHO, human Rhodopsin

mRHO, murine Rhodopsin

adRP, autosomal dominant *Retinitis Pigmentosa*

arRP, autosomal recessive *Retinitis Pigmentosa*

miRNA, micro RNA

AAV, adeno-associated virus

LCA, leber congenital amaurosis

hRHO-P, human rhodopsin promoter

ZF-ATF, zinc finger-based artificial transcription factors

DBD, DNA-binding domain

NLS, nuclear localization signal

RSC, retinal stem cells

eGFP, enhanced green fluorescent protein

GC, genome copies

PDE6, phosphodiesterase-6

Cnga, cGMP-gated channel

RHOK, rhodopsin kinase

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ABSTRACT

Viral-mediated gene therapy holds great promise for the treatment of severe inherited retinal diseases, such as *Retintitis Pigmentosa* (RP), which is caused by mutations in genes preferentially expressed in photoreceptor cells. The availability of vectors derived from the small adeno-associated virus (AAV) which efficiently and stably transduce the retina of animal models after intraocular administration strongly support the possibility to develop novel strategies for the treatment of such severe retinal degenerations otherwise incurable thus far.

The main goals of my PhD project were:

- generate artificial transcription repressors (ZFPs) targeted to the human rhodopsin promoter to silence at the transcriptional level the rhodopsin gene;
- assess the efficacy of the treatment and the impact on the disease progression in the RP mouse model.

Retinitis pigmentosa is by far the most studied inherited retinal disease. It is clinically and genetically heterogeneous recognizing autosomal recessive (arRP), autosomal dominant (adRP), X-linked, and digenic patterns of inheritance. More than 30 diseases genes have been identified so far and 12 of these have been associated with (adRP), representing between 15% and 35% of all cases. Despite recent success of the gene-based complementation approach for genetic recessive traits, the development of therapeutic strategies for gain-of-function mutations poses great challenges. General therapeutic principles to correct these genetic defects mostly rely on post-transcriptional gene regulation (RNA silencing). Engineered zinc finger protein (ZFP)-based-

repression of transcription may represent a novel and alternative mutation independent therapeutic approach for treating gain-of-function mutations, but proof-of-concept of this use is still lacking. In my PhD project we used a novel strategy to treat adRP based on zinc-finger-based artificial transcription factors (ZF-ATFs). These molecules can be engineered to silence genes carrying gain-of-function mutations that cause toxic effects into the cell where they are expressed. We generated ten artificial transcriptional repressors targeted to the human Rhodopsin which is the gene most commonly associated with adRP (20–30% of cases) with more than 150 mutations identified throughout its sequence, representing the most commonly mutated gene in RP.

We characterized *in vitro* the ability of artificial transcriptional repressors to bind specifically the human rhodopsin promoter in order to exert a specific transcriptional control and we selected two out of ten functional zinc-finger-based repressors of rhodopsin. One of this was selected as the most efficient and was enclosed in an AAV2/8 for *in vivo* experiments. We demonstrated that the selected artificial zinc-finger-based repressors (ZFRs) resulted in a robust transcriptional repression of hRHO impacting disease progression in a mouse model of adRP over-expressing the P347S mutation.

The data obtained support the use of ZFP-mediated silencing as a potentially relevant therapeutic strategy to treat gain of function mutations.

Introduction

INTRODUCTION

1. THE RETINA AND INHERITED PHOTORECEPTOR DISEASES

1.1 Structure of the eye and the retina

The eye is perhaps the most important sensory organ for humans and it is the first component of the visual system which allows assimilating information from the environment. This ability is called "Visual Perception" and it is by far the most complex sensory system. This is due to the fact that vision must handle demands such as the transduction of light stimuli to neural impulses, the binocular and more distant depth perception and the colour discrimination.

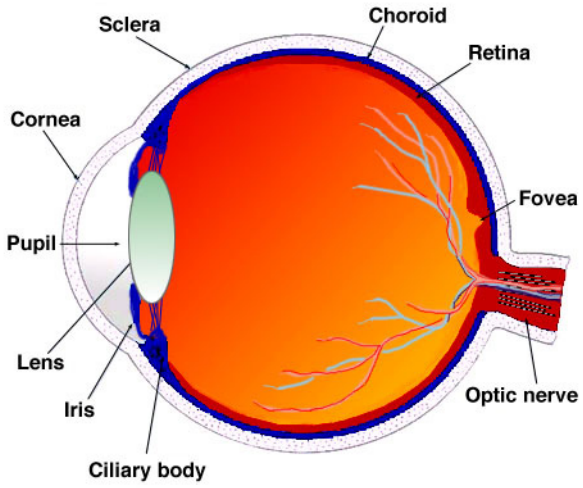

Figure 1. Schematic view of the human eye anatomy

The structure of the human eye (**Fig. 1**) can be divided into three main layers or tunics whose names reflect their basic functions: the fibrous, the vascular, and the nervous tunics. The fibrous tunic, also known as the *tunica fibrosa oculi*, is the outer layer of the eyeball consisting of the cornea and sclera. It consists of dense connective tissue filled with the collagen to both protect the inner components of the eye and maintain its shape. The vascular tunic, also known as the *tunica vasculosa oculi*, is the middle vascularized layer, which includes the iris, ciliary body, and choroids. The iris sits between the anterior chamber which contains the aqueous humour

essential for nourishing the lens and the cornea, and the posterior chamber filled with the vitreous humour. This substance is jelly-like and, besides helping the eye to keep its shape, it transmits the light to the back of the eye. The lens is a clear, flexible structure responsible for sharpening of the image at the retina and it is connected to the ciliary body (which contains the ciliary muscles). The choroid contains blood vessels that supply the retinal cells with necessary oxygen and remove the waste products of respiration. The nervous tunic, also known as the *tunica nervosa oculi*, is the inner sensory structure, which includes the retina.

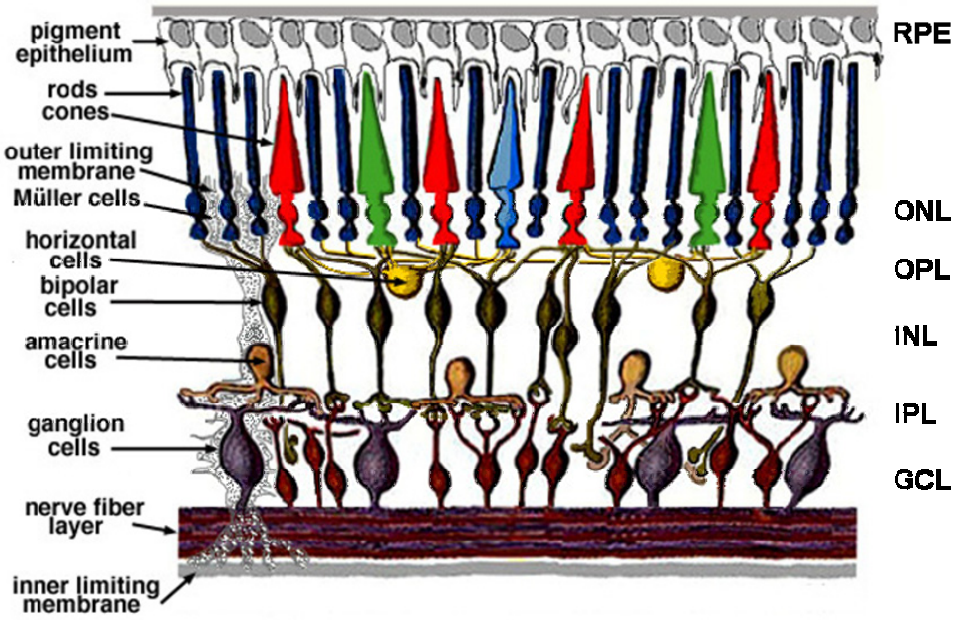

Figure 2. Drawing depicting the layered structure of the different retinal cell types

Retina is a light sensitive tissue lining the inner surface of the eye (**Fig. 2**). It is a highly organized array of neurons, which serve as transducer for the conversion of the light into neuronal signals which eventually reach the brain. Due to the complexity of this process, retinal cells give rise to a variety of neuronal cell types, which conduct and facilitate the entire cascade of events. Retina is composed of seven classes of cells structured in layers: photoreceptor

cells (rods and cones), bipolar cells, horizontal cells, amacrine cells, Müller glia cells and retinal ganglion cells (**Fig. 2**).

The most outer layer is the retinal pigment epithelium (RPE), which is situated between the choroid and the photoreceptors. RPE nourishes the retina and is involved in the phagocytosis of the outer segment of photoreceptor cells and is also involved in the chromophore regeneration (see section 1.3). The second layer, the photoreceptor layer, comprises the outer and the inner segments of photoreceptors (rods and cones), while the photoreceptors cell bodies form the outer nuclear layer (ONL). At the synaptic terminals of photoreceptors, in a region called the outer plexiform layer (OPL) light induced signals are transferred from rods and cones to bipolar and horizontal cells which together with Müller glia and amacrine cells form the inner nuclear layer (INL). Horizontal cells provide lateral interaction in the OPL and aid in signal processing. One type of rod bipolar cells and at least 10 different types of cone bipolar cells transfer light-induced signals to the inner plexiform layer (IPL), which comprises dendrites of amacrine cells and ganglion cells. Amacrine cells are inhibitory interneurons; there are about 40 different subtypes. Müller cells are the main glial cells of the retina. They form architectural support structures stretching radially across the thickness of the retina and set the limits of the retina at the outer and inner limiting membranes, respectively. Dendrites of amacrine cells, bipolar and ganglion cells form the inner plexiform layer (IPL). The ganglion cell layer (GCL) forms the innermost retinal layer. Ganglion cell dendrites collect the signals of bipolar and amacrine cells and transmit these signals through their axons, which form the optic nerve, to the visual centres of the brain[1].

1.2 The photoreceptor cells

Perception of light initiates in the highly specialized retinal cells called photoreceptors (**Fig. 3**). Photoreceptors are highly polarized retinal neurons with the unique property of transforming physical signals (photons of light) first into biochemical messages and then into electrical message "perceived" by specialized brain structures (visual cortex). Photoreceptors contain four distinct compartments: the outer segment (OS), a thin cilium, which connects the outer to the inner segment (IS), a cell body containing the nucleus and the cytoplasm and a short axon connecting the photoreceptor cell to interneurons (**Fig. 3**). The OS is the compartment in which the conversion of light energy into electrical signals (phototransduction cascade) occurs. It consists of an array of flat membranous disks that arise during development as a series of invagionations of the cell's plasma membrane. These discs disintegrate near the apical surface of the cells and the cellular debris are removed through phagocytosis by the adjacent RPE[2] following a diurnal rhythm. The old discs are gradually replaced by newly formed ones that migrate to the base of the OS[3]. The IS contains most of the photoreceptor metabolic machinery, including the ER, the Golgi apparatus, and the mitochondria. Cellular components and metabolites are exchanged and/or transported between the IS and OS through the narrow connecting cilium.

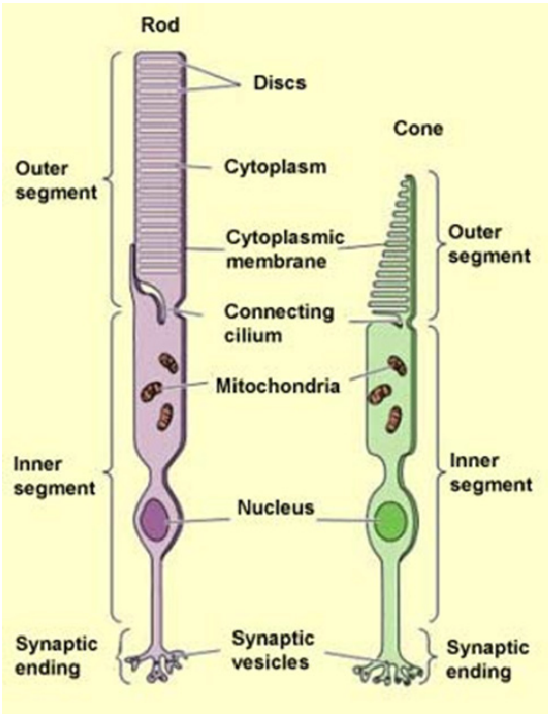

Figure 3. Structure of the photoreceptor cells: rod (left) and cone (right)

There are two types of photoreceptor cells in the human retina: rods which mediate dim-light vision and cones which function in bright light. Rods represent 95% of photoreceptor cells in the human retina and are responsible for sensing contrast, brightness and motion. They contain a photopigment called rhodopsin (RHO, max absorbance 500 nm) which is capable of trapping photons. The cones perceive fine resolution, spatial resolution, and colour vision and they contain three different colour pigments, blue-sensitive pigment (445 nm), green-sensitive pigment (535 nm) and red-sensitive pigment (570 nm) sensitive to different colours: blue, green and red respectively[1]. In humans, cone density is maximal in the fovea, which contains about 10% of the cones of the retina; their density decreases drastically across the macula, beyond the borders of which density is relatively constant but asymmetric, with higher densities on the nasal side of the retina. Rods are also distributed unevenly across the retina: there are no rod photoreceptors within of the fovea. Beyond this rod-free zone, they increase rapidly to reach a peak along an elliptical ring at the eccentricity of the optic disc. In rodents, fovea is not present and photoreceptors are constantly distributed through the retina.

1.3 The phototransduction cascade

Transduction of absorbed light into electrical signal that is eventually perceived as sight takes place within photoreceptor cells via a complex molecular process called phototransduction[4]. Rod phototransduction has been more comprehensively studied than cones one. It takes place in the rod OS discs with the absorption of light by rhodopsin which is a photopigment that counts for 80% of total amount of rod outer segment proteins. It is composed of a backbone, termed rod-specific opsin, a seven transmembrane G-protein-

coupled receptor, bound to the light-sensitive chromophore 11-*cis*-retinal (11cRAL) a derivative of vitamin A[5]. The phototransduction cascade initiates when the visual pigment, rhodopsin, absorbs a photon (hv; **Fig. 4**). This induces a conformational change of the chromophore to all-*trans*, which still has the

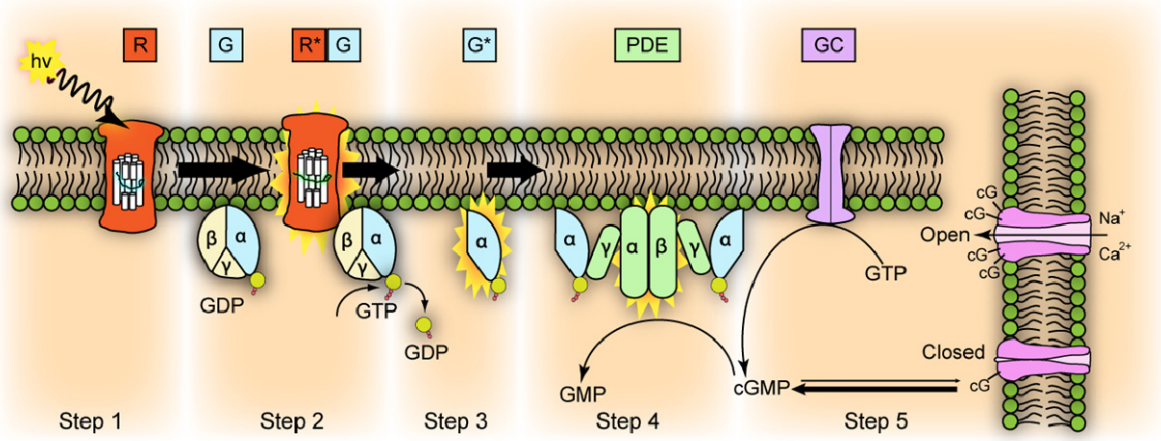

Figure 4. Phototransduction pathway in rod photoreceptors. R*, photoactivated rhodopsin; G, transducin; PDE, phosphodiesterase 6; GC, guanylate cyclase. See text for details.

same chemical structure as the *cis* but a different physical form. Because the all-*trans*-retinal (atRAL) no longer fits with the rhodopsin, it begins to pull away from it until there is a complete split (within seconds). This in turn induces conformational changes in the rhodopsin which is activated (R*). This intermediate molecule (metarhodopsin II) interacts with the next member of the cascade, a G-protein called Transducin (G) which is an heterotrimeric protein ($\alpha\beta\gamma$). This interaction induces the α subunit of the transducin to exchange a bound guanosine diphosphate (GDP) moiety to guanosine triphosphate (GTP). The activated GTP-bounded α subunit (G*α) detaches from the β and γ subunits of the transducin and associated with the next member of the cascade, the phosphodiesterase 6 (PDE6) which is a multisubunit complex, composed of two tightly bound catalytic subunits, α (99kDa, αPDE) and β (98kDa, βPDE) in addition to two identical inhibitory γ subunits of 11kDa. The enzyme is anchored

to the rim membrane by an isoprenylic group at the C-terminus of the α and β subunits. The G*α subunit interacts directly with the inhibitory γ subunit of the PDE6. An increase in the activity of the cGMP phosphodiesterase at this stage induces a fall in the concentration of cGMP in the cytoplasm[5] which leads to the closure of cGMP-gated cation (Na^+ and Ca^{++}) channels on the plasma membrane of the rod's OSs with a consequent decline in calcium concentration within the cell. This causes a graded hyperpolarization of the plasma membrane which is conveyed, as in all neuronal cells, to the synaptic region of the rod where it decreases the amount of neurotransmitter (glutamate) release. This initial signal is transmitted via second-order retinal neurons to the optic nerve and to the brain. The decline in calcium concentration mediates the recovery of the photoreceptor cell after a bleach of light. This is as important for maintaining sensitivity in vision as the cell's ability to respond to a single photon. Deactivation of rhodopsin starts with phosphorylation by rhodopsin kinase (RHOK) and is followed by the capture of rhodopsin by the protein arrestin[6]. The arrestin-binding prevents further activation of transducin and releases the all-*trans*-retinal from rhodopsin. The concentration of cGMP within the cell is restored by the increased synthesis of cGMP by a retinal guanylate cyclase (GC). These pathways are triggered by the decline in the intracellular calcium concentration and mediated by a family of calcium-binding proteins, including recoverin and guanylyl cyclise activating protein (GCAP). The calcium-bound recoverin inhibits the activity of rhodopsin kinase[7]. Sustained phototransduction depends on replenishing the 11-*cis*-retinal lost as a result of light activation of the visual pigments in a pathway which takes place between the photoreceptor cells and the RPE in a process called visual cycle[8].

1.4 The visual cycle pathway

Absorption of a photon of light by rhodopsin causes isomerisation of the chromophore from 11-*cis*-retinal to all-*trans*-retinal. In order to restore light sensitivity of rhodopsin all-*trans*-retinal must be converted back to 11-*cis*-retinal through a multistep pathway called visual cycle (**Fig. 5**). Visual cycle initiates in

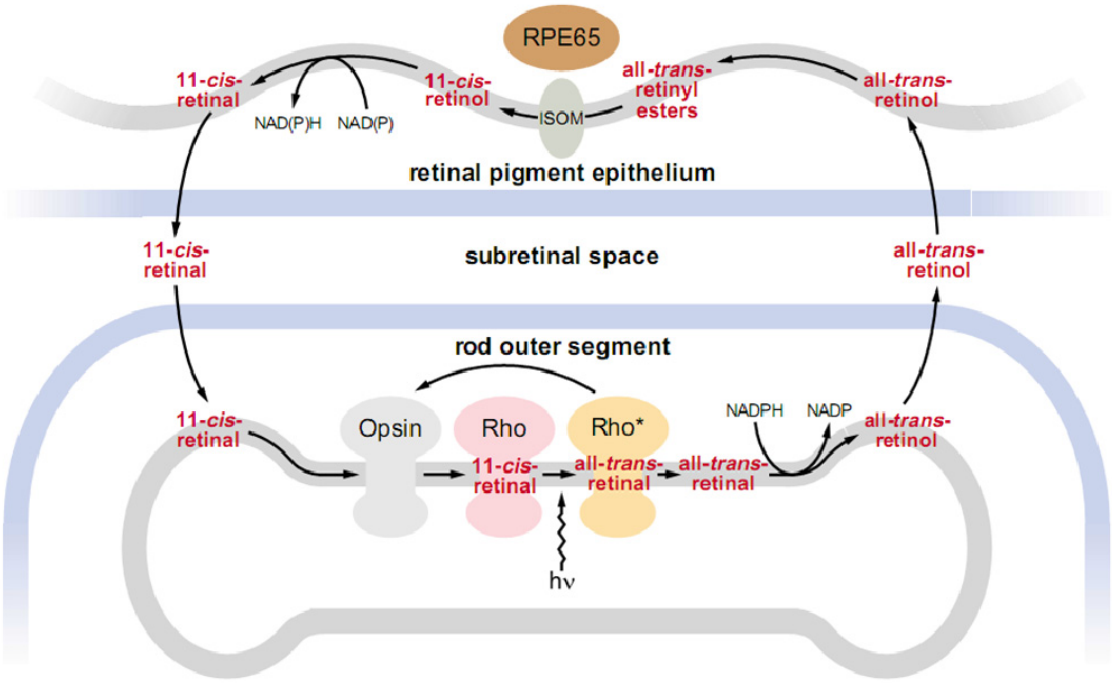

Figure 5. Rhodopsin regeneration during visual cycle, a process wich begins in the rod outer segments and is completed in the retinal pigment epithelium.

the photoreceptor cells, precisely in the inner surface of the rod disks membrane, with the release of all-*trans*-retinal which is subsequently transferred to cytoplasmic surface of the disks by retina-specific ATP-binding cassette transporter (ABCA4)[9; 10]. In the photoreceptor cytoplasm, all-*trans*-retinal is reduced by an all-trans retinol dehydrogenase (atRDH) and the resulting all-*trans* retinol (vitamin A) is transported across the subretinal space to the RPE. Within the RPE, all-*trans*-retinol is bound to cellular retinal binding protein (CRBP)[11] and immediately esterified to all-*trans*-retinyl esters (atRE) by

lecithin retinol acyltransferase (LRAT)[12]. atRE is isomerized to 11-*cis*-retinol by isomerohydrolase (IMH) in conjunction with RPE65 protein. The resulting 11-*cis*-retinol (11cROL) is oxidized by the retinol dehydrogenase (11cisRDH) to the final product 11-*cis* retinal[13]. The 11-*cis*-retinal exits the RPE, traverses the subretinal space, and enters the photoreceptor outer segments where it combines with opsin protein to form a new molecule of light-sensitive rhodopsin.

1.5 Inherited retinal diseases

The complex retinal structure and signalling network, which includes numerous neurotransmitters, neuromodulators, phototransduction proteins, transcription factors, etc., lead to a wide range of targets for potential events that may cause pathogenic changes in its function. According to some estimates[14] the eye is the fourth system most commonly affected by genetic diseases in humans. At the same time, genetic eye diseases, both monogenic and genetically complex, comprise the commonest causes of blindness in children and adults worldwide[15]. Out of different eye diseases, retinal disorders are especially important since there are more then 100 diseases that include a form of retinal dystrophy, as listed in the online database of human genetic diseases (Online Mendelian Inheritance in Man-OMIM). In the industrialized world, the most common diseases involving the retina are diabetic retinopathy, glaucoma, and age-related macular degeneration (AMD), which together affect several percent of the population[16]. Each of these diseases has both genetic and non-genetic components. Contrary to complex diseases, the simple Mendelian retinal diseases have an earlier onset and some have a more severe clinical course than typically observed for the three more common disorders listed above and,

for the most part, they are untreatable. These characteristics, together with the possibility of exploiting genetic approaches to understand disease mechanisms, have drawn attention on the Mendelian disorders. *Retinitis pigmentosa* is by far the most studied inherited retinal degeneration and it may serve as an example of remarkable genetic heterogeneity connected with retinal dystrophies.

1.6 *Retinitis Pigmentosa*

Retinitis Pigmentosa (RP) is the term given to a set of inherited retinal degeneration with a prevalence of 1:3500 worldwide[17; 18] neither preventable nor curable[19; 20]. It is an highly variable disorder, indeed some patients develop symptomatic visual loss in childhood whereas others remain asymptomatic until mid-adulthood. Many patients fall into a classic pattern of difficulties with dark adaptation and night blindness in adolescence and loss of mid-peripheral visual field in young adulthood. As the disease advances, they experience a drop of far peripheral vision which, develops in tunnel vision and eventually in central vision lost, usually by age 60 years[20]. Visual symptoms indicate the gradual loss of the two photoreceptor types: first loss of rods, which mediate achromatic vision in starlight or moonlight and are more abundant in the peripheral retina, then loss of cones, which are important for colour vision and fine acuity in daylight. Most patients are legally blind by age 40 years because of severely constricted visual field. In most form of RP, loss of rod function exceeds reduction of cone sensitivity. In other types, rod and cone decline is similar. Occasionally, the deficit of cones far exceeds that of rods; in this case the disease is termed cone-rod degeneration, and loss of visual acuity and defective colour vision are the prominent early symptoms.

1.6.1 Diagnosis of RP

Clinically, RP patients are diagnosed based on three main abnormalities: atrophy and pigmentary changes of the retina and RPE, abnormal electroretinogram (ERG) and attenuation of the retinal vasculature and changes to the optic nerve head[18]. In addition, reduction of the visual field, colour vision impairment, dark adaptation or cataracts, which is showed in about 50% of individuals with RP, can be observed. Pigmentary changes in the retina are evident during the fundus examination and remain a common factor in RP diagnosis (**Fig. 6**); they mainly consist in marked pigment epithelial thinning, optic disc pallor and the classical "bone spicule deposits which result from the release of pigment by degenerating cells in the retinal pigment epithelium. The pigment granules accumulate in perivascular clusters, known as "bone-spicule formations" due to their morphological appearance, in the neural retina. Consequently, early in the disease, the pigmented posterior pole of the eye, the fundus, develops a granular appearance. This is followed by the development of bone-spicule pigmentary deposits overlying the depigmented fundus[21]. The main tool for diagnosis and classification of retinitis pigmentosa is the Electroretinogram (ERG) which is an objective measure of retinal function and it is useful for accurate diagnosis of disease, for assessment of severity, to follow the course of the disease, to provide a visual prognosis and for measurement of responses to treatments. In this procedure, retina is either dark adapted

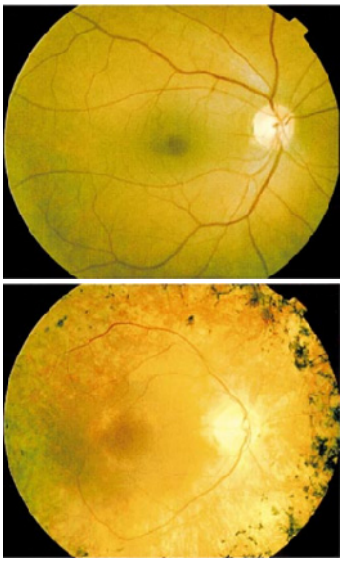

Figure 6. Fundus photograph of an healty individual (upper panel) and of a patient with RP (lower panel).

(scotopic ERG) or adapted to a specific level of light (photopic ERG), and then stimulated with a brief flash of light. The summed electrical response of the retina is recorded extraocularly with a contact lens electrode. A single-flash dim blue light elicits a rod response, a brighter single-flash white light elicits a combined rod-plus-cone response, and flickering (30Hz) white stimuli generate cone-isolated response. With single flashes (0-5 Hz) of white light, an initial a-wave shows hyperpolarisation of photoreceptors and a subsequent b-wave result from depolarisation of cells in the inner nuclear layer. Typically, patients with RP have reduced rod and cone response amplitudes and a delay in their timing[18] (**Fig. 7**).

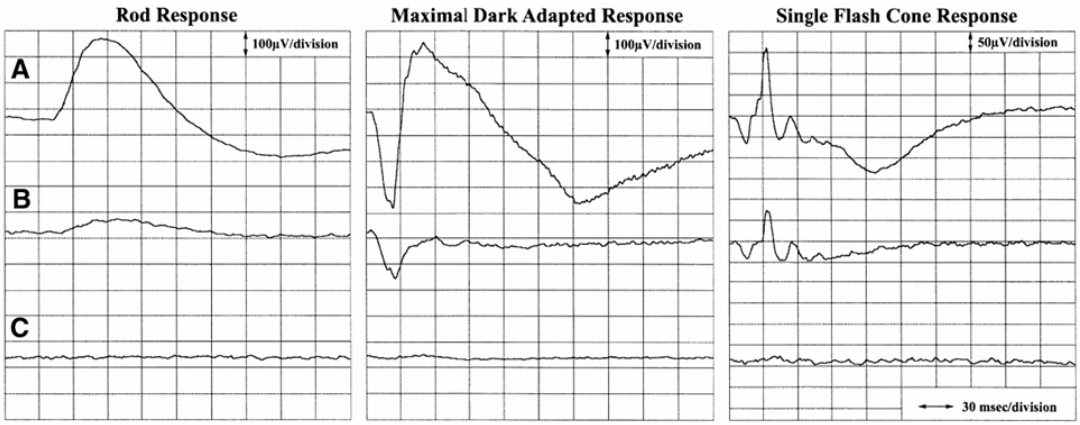

Figure 7. Rod-isolated, combined rod and cone responses to the maximal intensity flash presented in the dark-adapted state and flash light-adapted cone responses are shown from (**A**) a normal individual, (**B**) a patient with moderately advanced RP and (**C**) a patient with advanced RP.

1.6.2 Genetic of *Retinitis Pigmentosa* (RP)

Retinitis Pigmentosa is due to many distinct causes and involves diverse biological pathways but with overlapping symptoms and similar consequences. Most forms of RP are monogenic and can be inherited with classical patterns[18; 22]: autosomal dominant (adRP) forms account for 30-40% of total cases, autosomal recessive (arRP) forms are the most common variants with a

frequency of 50-60% while X-linked (X-lRP) can be observed in 5-15% of affected individuals. However, some unusual patterns as digenic inheritance[23] or maternal (mitochondrial) inheritance[24] have also been reported in the literature. In most cases, patients with *Retinitis Pigmentosa* have no associated systemic or extraocular abnormalities. Moreover, there are multisystem diseases in which RP is accompanied by involvement of other tissues and organs. Examples are Usher's syndrome (USH), in which *Retinitis Pigmentosa* is associated with hearing impairment and it is the most frequent syndromic form (10-20% of total cases)[25], or Bardet-Biedl syndrome, where RP is associated with obesity, cognitive impairment, polydactyly, hypogenitalism, and renal disfunction[26]. A remarkable feature of *Retinitis Pigmentosa* is its high genetic heterogeneity: so far, more than 50 genes responsible for non-syndromic forms have been identified that accounts for 60% of total cases[20] (RetNet: http://www.sph.uth.tmc.edu/Retnet/) while about 40% of cases are due to genes not yet mapped. Mutations in genes preferentially expressed in photoreceptors are the most common cause of RP followed by RPE-specific genes. In rare cases RP is caused by mutations in genes expressed in other retinal cell types or outside the eye. Despite the genetic heterogeneity of RP, photoreceptor cells mainly degenerate by apoptosis[27] although the mechanisms by which the genetic defect leads to cell death are still unclear[28]. According to the known or presumed function of the encoded proteins, the genes responsible for RP so far identified have been clearly grouped into functional categories as schematically depicted in **Table 1**[20]. Some of these genes encode proteins in the rod photoreceptor cascade, the specific biochemical pathway that transduces light stimuli and leads to changes in photoreceptor-cell polarisation. Recessive null mutations in any of these genes would evidently interfere with

rod function and produce night blindness from birth. Subsequent death of rod photoreceptors is probably an outcome of the deranged physiology associated with the defective or absent gene product. For example, without functional rod cGMP phosphodiesterase, arising with recessive defects in PDE6α or PDE6β, cGMP concentrations in rod photoreceptor outer segment rise and this in turn opens cGMP-gated channels in the plasma membrane. Rods apparently die

	Inheritance
Phototransduction cascade	
RHO, rhodopsin (G-protein coupled photon receptor)[114]	Dominant, recessive
PDE6A, rod cGMP-phosphodiesterase α subunit (G-protein effector enzyme)[115,116]	Recessive
PDE6B, rod cGMP-phosphodiesterase β subunit (G-protein effector enzyme)[115,116]	Recessive
CNGA1, rod cGMP-gated cation channel α subunit[117]	Recessive
CNGB1, rod cGMP-gated cation channel β subunit[118-120]	Recessive
SAG, arrestin (rhodopsin deactivation)[121]	Recessive
Vitamin A metabolism	
ABCA4, ATP-binding cassette protein A4 (photoreceptor disc membrane flippase for vitamin A)[122,123]	Recessive
RLBP1, retinaldehyde binding protein (11-cis-retinaldehyde carrier)[124]	Recessive
RPE65, (vitamin A trans-cis isomerase)[125,126]	Recessive
LRAT, lecithin retinol acetyltransferase (synthesises vitamin A esters)[125]	Recessive
RGR, RPE-vitamin A G-protein coupled receptor (photon receptor in RPE)[127]	Recessive
Structural or cytoskeletal	
RDS, peripherin (outer disc segment membrane protein)[128,129]	Dominant, digenic
ROM1, rod outer segment protein[130]	Digenic
FSCN2, fascin (actin bundling protein)[131,132]	Dominant
TULP1, tubby-like protein 1[133]	Recessive
CRB1, crumbs homologue (transmembrane protein, adherent junctions)[134]	Recessive
RP1, microtubule-associated protein (microtubule formation and stabilisation)[135]	Dominant, recessive
Trafficking of intracellular proteins	
MYO7A, myosin 7A (melanosome motility protein)[148]	Recessive
USH1G, scaffold protein containing ankyrin repeats and SAM domain (Usher's type I protein traffic regulator)[149]	Recessive
	(Continues on next page)

Table 1. Genes for *retinitis pigmentosa* and functions of their protein products (continues on next page).

(Continued from previous page)	
Maintenance of cilia/ciliated cells (possible role in intracellular trafficking)	
BBS1, Bardet-Biedl syndrome 1[150]	Recessive
BBS2, Bardet-Biedl syndrome 2[150-152]	Recessive
ARL6, ADP-ribosylation factor like 6[150]	Recessive
BBS4, Bardet-Biedl syndrome 4[150,153]	Recessive
BBS5, Bardet-Biedl syndrome 5[150,154]	Recessive
MKKS, McKusick-Kaufman syndrome[150,155]	Recessive
BBS7, Bardet-Biedl syndrome 7[150,156]	Recessive
TTC8, tetratricopeptide repeat domain 8[150,156,157]	Recessive
PTHB1, parathyroid hormone-responsive B1 gene[150]	Recessive
RPGR, trafficking of proteins in the cilia [158,159]	X-linked
pH regulation (choriocapillaris)	
CA4, carbonic anhydrase IV (carbon dioxide/bicarbonate balance)[160]	Dominant
Phagocytosis	
MERTK, mer tyrosine kinase proto-oncogene (RPE receptor involved in outer segment phagocytosis)[161]	Recessive
Other	
CERKL, ceramide kinase-like (ceramide converting enzyme)[162]	Recessive
IMPDH1, inosine-5′ monophosphate dehydrogenase type I (guanine nucleotide synthesis)[163]	Dominant
BBS10, vertebrate-specific chaperonin-like protein[29]	Recessive

Table 1. Genes for *retinitis pigmentosa* and functions of their protein products.

from the rush of cation flowing into the cells through these open channels[29]. Another relevant example is given by dominant mutations in rhodopsin gene which are probably detrimental to rods because the mutant forms of the protein are toxic to rod photoreceptors. The toxic effects are attributable to interference with metabolism, perhaps by formation of intracellular protein aggregates, from a defect in intracellular transport, or from a fault in the structure of the photoreceptor outer segments[30]. The reason why mutations in genes exclusively expressed in rod photoreceptors cause the death of both rod and cone cells is not yet clear. The secondary death of cones might be due to reliance on neighbouring rods for survival. The discovery of the RdCVF protein,

a factor released from rods that promote cone survival, provides a possible explanation to this question[31]. The second most common group of genes mutated in RP are involved in visual cycle, in particular in recycling the rhodopsin chromophore 11-*cis*-retinaldehyde[18]. An example is the gene *RPE65* which is primarily expressed in the RPE and endowed with isomerise activity for the rhodopsin ligand 11-*cis*-rtinal[32]. Mutations in genes encoding photoreceptor structural proteins or transcription factors have also been identified. Moreover, some genes for RP are expressed in tissue outside the eye, and others encode proteins that are essential for life. For example, non syndromic RP is caused by dominant mutations in genes PRPF31, PRPF8 and PRPF3 that encode components of the spliceosome, a vital complex that excises introns from RNA transcript[33].

1.6.3 RP due to rhodopsin mutations

Mutations in the rhodopsin gene are the most common cause of *Retinitis Pigmentosa* among human patients and account for 20-30% of adRP cases[34-37] (**Fig. 8**). Rhodopsin is the light-absorbing protein that mediates vision at low light levels. Like other visual pigments, it consists of a chromophore (11-*cis*-retinal) covalently bound to an integral membrane protein (opsin). Photoisomerization of retinal from 11-*cis* to all-*trans* induces a conformational change in the apoprotein, leading to a conformation that is competent to activate the photoreceptor-specific G-protein transducin, thereby initiating the phototransduction cascade. In mammals, rhodopsin accumulates to a level of $5x10^7$ molecules/rod outer segment and is synthesized throughout life at a rate of $5x10^6$ molecules/rod/day[38]. Rhodopsin is localized to the rods outer segments which contain hundreds of flattened

membrane sacs (also called disks) stacked in close apposition and where it represents the 80% of total proteins content[39]. Over 150 mutations in the rhodopsin gene leading to autosomal dominant RP (adRP) have been characterized so far which can be grouped in two distinct classes of biochemical defect. Approximately 85% of the mutant proteins (class II) occurs in the N-terminus of the protein and are produced at lower levels than the wild

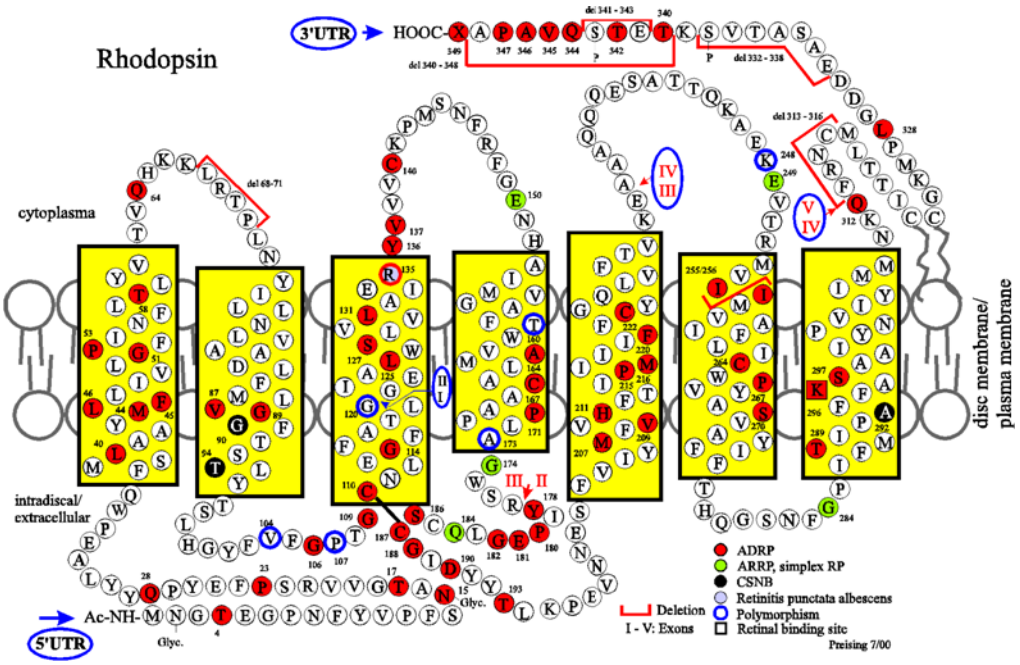

Figure 8. Structure of the rhodopsin protein with the most common mutations highlited.

type, accumulate predominantly in the endoplasmic reticulum, and bind 11-*cis*-retinal variably or not at all. This class of proteins appears to be defective in folding and/or stability. The remaining 15% of mutant proteins (class I) map very close to the C-terminus, a region of the protein for which no function has yet been assigned, and resemble the wild type protein, indeed are produced at high levels, accumulate in the plasma membrane and efficiently bind to 11-*cis*-retinal to form photolabile pigments[40; 41]. Because these rhodopsin mutations were identified in the heterozygous condition in patients with adRP, it seems

likely that the mutant proteins interfere with or participate aberrantly in some physiological process. A second possibility is that they are physiologically silent and that RP results from haploinsufficiency, a scenario that seems unlikely in light of the finding that heterozygous carriers of one apparently null mutation in the rhodopsin gene do not have RP[42]. Such mutational heterogeneity represents a significant barrier to the development of therapies for adRP associated with rhodopsin gene.

1.6.4 Current treatment protocols for RP

At the moment, no cure is available for RP but some pharmacological treatments are recommended by clinicians to slow visual loss. Nutritional or neuroprotective treatments that affect secondary biochemical pathways have the advantage of being less dependent on the disease-causing mutation and could therefore be widely applicable.

For example, based on a study of the natural course of *Retinitis Pigmentosa*, patients assuming vitamin A, vitamin E, or both were recorded to have slower declines in ERG amplitudes than those not taking such supplements[43]. This observation prompted a randomised clinical trial of oral vitamin A and E supplements in 601 patients with dominant, recessive and X-linked non-syndromic *Retinitis Pigmentosa* and Usher's syndrome type II. Patients assigned high-dose vitamin A showed a significantly slower decline in cone ERG amplitudes than did those in the other groups. Based on these results, many clinicians recommend that adults with early or middle stages of *Retinitis Pigmentosa* take 15,000 IU of oral vitamin A palmitate every day and avoid high dose vitamin E supplements. Toxic effects have not been reported, even if older individuals could also be monitored for bone health because a slight increased

risk for hip fractures due to osteoporosis has been reported in postmenopausal women and men older then 49 years who take vitamin A supplements[43]. Another nutritional treatment assessed for patients with *Retinitis Pigmentosa* is docosahexaenoic acid (DHA), an omega-3 fatty acid found in high concentration in oil fish. DHA is apparently important for the photoreceptor membrane, since membranes containing rhodopsin and cone-opsin in photoreceptor cells have very high dose of this fatty acid. Amounts of DHA in red-blood cells are on average lower in patients with RP than in unaffected people. Nevertheless, results from two independent studies of oral DHA supplements to individuals with *Retinitis Pigmentosa* did not show clear benefits even if people with the highest concentrations of DHA in red-blood cells had the slowest rates of retinal degeneration[44].

Further, finding of work done in animals have shown that some neurotrophic factors can promote photoreceptor survival[45; 46]. Results of a human phase I study of an intravitreal capsule containing cells that release ciliary neurotrophic factor have been reported. Small-molecule drugs are also being assessed as possible treatments for RP. For example, in a study of a calcium-channel blocker (diltiazem), researchers claimed a beneficial effect in a mouse model of RP due to recessive mutations in the β-subunit of rod phosphodiesterase. However, three subsequent trials of this drug in mice and other animal models by independent groups failed to confirm a benefit[47; 48].

Lastly, nanotechnology and biotechnology leading company make great effort in order to develop devices to electrically stimulate the retina, optic nerve, or visual cortex. The first results obtained are encouraging since the few people testing the first version of these devices have reported seeing phosphenes (flash of light) in response to direct retinal stimulation[49].

It’s anyway clear that, increasing the knowledge of the biochemical defects associated to RP is essential to develop diverse approaches for the treatment of such challenging disease. Nevertheless, these data show that the above-mentioned approaches cannot be considered a real therapeutic option to halt or reverse RP thus far.

2. GENE THERAPY FOR RETINAL DISEASES

2.1 Gene therapy and the retina

Gene therapy aims at delivering corrective genetic material to a cell, tissue or target organ in order to prevent or cure a disease[50]. Nucleic acids do not readily cross cell membranes; consequently, it is necessary to envelope the genetic material in a lipidic-containing complex or incorporate it in a viral vector[51; 52]. The former inserts genetic material directly into the target cells by fusing to the host cell membrane. Unfortunately they are not selective for a target cell, have low transduction efficiency and mediate short-lived gene expression[51; 52]. On the other hand, viral delivery of therapeutic genes appears much more promising since a prolonged transgene expression is obtained with the use of particular recombinant viral vectors[51].

The retina represents an ideal target organ for gene therapy approaches for a number of reasons: i) it is easy to manipulate and the small size of the eye allows the use of low doses of vector; ii) the partial immune privileged properties of the eye can limit immune responses toward the transgene and the vector; iii) the eye is enclosed and the presence of the blood–retinal-barrier, of the RPE and of the intracellular junctions in the inner retina can help avoiding unintentional spreading of vectors to neighbouring tissues as well as to the general circulation; iv) non invasive *in vivo* techniques for ocular tissues imaging and visual function evaluation are available (Electroretinogram, ERG; Optical Coherence Tomography, OCT); v) since ocular diseases develop bilaterally and symmetrically, if one eye is treated the other can be used as an useful untreated internal control; vi) surgical procedures have been adapted for

the transfer of genetic material into the two main ocular compartments (Subretinal and intravitreal).

2.2 Gene-based approaches to treat inherited retinal degenerations

Gene-therapy approaches are dependent on the type of mutation to reverse thus different strategies may be adopted in order to treat a gain- or a loss-of-function mutation as well as for the treatment of a dominant-negative mutation or in the case of haploinsufficiency. However, such strategies can be divided in two distinct groups: gene replacement or gene silencing strategies.

2.2.1 Gene replacement strategy

Recessively inherited diseases typically result from alterations that eliminate the encoded protein (loss-of-function mutations). They can give rise to dominant inheritance if the remaining normal copy does not express sufficient protein to meet cell's needs. Dominance resulting from inadequate expression levels is known as haploinsufficiency and it is extremely rare, thus for most genes, one allele is sufficient to preserve retinal function. For the treatment of loss-of-function mutations, the introduction of a normal copy of the gene into the diseased tissue[53] (gene-replacement approach) can supply the missing protein. This approach, for example, has been applied to a form of Leber Congenital Amaurosis (LCA)[54], the most severe form of inherited childhood blindness. The target gene in this case is *RPE65*, which is expressed in the RPE and encodes for an isomerise that is essential for production of the photopigment 11-*cis*-retinal[32]. Subretinal administration of an AAV vector containing a corrected copy of the *RPE65* gene has shown to restore vision in mice and dogs harbouring a mutation in the gene[55-61]. The recent success of clinical trials for the treatment

of this disease is providing sound evidence for the use of gene-based complementation therapeutic strategy to treat genetic recessive traits[62-68]. Gene-replacement approach has also been successfully used in animal models of genetically identified forms of RP, however, in this case, it is essential to intervene during early stages of the diseases progression in order to prevent the loss of photoreceptor cells.

2.2.2 Gene silencing strategy

Dominant mutations typically alter the transcribed amino acid sequence and result in toxic variants of the encoded protein (gain-of-function of dominant-negative mutations). One strategy to treat these alterations is to eliminate the mutant gene (gene silencing approach) and hope that the remaining normal copy of the gene will provide sufficient functional protein. The main target of genetic silencing strategies is the mRNA transcript whose function can be inhibited by antisense-, ribozyme- and more recently by small interfering RNAs-(siRNAs) and miRNA-based approaches. In particular, RNAi is holding great promise for treating dominant diseases for its efficiency in mRNA transcripts cleavage in animal models of adRP due to P23H or P347S[53; 69-74]. An emerging alternative to such RNA-targeting approaches is the modulation of gene expression at the transcriptional level by using artificial zinc finger-based transcription factors (ZF-TFs) or the genomic inactivation of a disease gene by using targeted zinc finger-based nucleases (ZFNs)[75-77]. The action of such novel approaches is targeted to the genomic sequence, silencing the downstream target gene independently by the causative mutation identified; as a consequence they allow the development of economic viable therapeutics since they circumvent the mutational heterogeneity associated to diseases,

such as adRP due to rhodopsin mutations. Indeed, as mentioned before, mutations in the rhodopsin gene are the most common cause of *Retinitis Pigmentosa* (RP) among human patients and nearly all rhodopsin mutations are dominant (The only characterized rhodopsin recessive allele is the mutation E249ter[42]).

2.3 Animal models of RP

The study of animal models with naturally occurring degenerative retinal diseases has been important for the discovery of numerous candidate genes, some of which have led to the identification of new disease genes in humans. These animals are useful models for understanding molecular pathways activated during the disease and to test experimental therapies for this otherwise incurable group of defects. Furthermore, the ability to generate gene-specific transgenic and targeted (knock-out or knock-in) mice, allows the creation of new models of human retinal disease and facilitate the elucidation of gene dysfunction. Several animal models of RP are available, mostly in rodents, pigs and canines[78-80]. Each of them shows peculiar phenotypical features depending on the mutated gene/allele, although all RP models are characterized by photoreceptors degeneration with the main differences consisting in the onset and time course of the disease. In this paragraph I will focus on the most common animal models of RP due to mutations in the rhodopsin gene. In 1997, Humphries and colleagues generated mice carrying a targeted disruption of the rhodopsin (*RHO*) gene[81]. The rod outer segments of Rho-/- mice do not develop fully and photoreceptor cells are lost over a 3 month period. No rod ERG response is present in 8-weeks-old animals. Rho+/- animals retain the majority of their photoreceptors although the inner and outer

segments of these cells display some structural disorganization and the outer segments become shorter in older mice. The rhodopsin knock-out animals provide a useful genetic background on which to express other mutant opsin transgenes as well as assessing the therapeutic potential of re-introducing functional rhodopsin genes into degenerating retinal tissues. The P23H mutation is the most prevalent mutation in human adRP patients in US[82]. Twelve percent of American patients with autosomal dominant *Retinitis Pigmentosa* (adRP) carry a substitution of histidine for proline at codon 23 (P23H) in their rhodopsin gene, resulting in photoreceptor cell death from the synthesis of the abnormal gene product. Transgenic rodents and pigs overexpressing the dominant P23H rhodopsin mutations that recapitulate the human disease have been generated[83-87]. Transgenic mice containing the P23H mutation appear to develop normal photoreceptors, but their light-sensitive outer segments never reach normal length[85]. With advancing age, both rod and cone photoreceptors are progressively reduced in number. The degeneration of the transgenic retina is associated with a gradual decrease of light-evoked electroretinogram responses. The disease mechanism associated with the P23H mutation remains unclear. Haploinsufficiency is unlikely, since humans and mice heterozygous for rhodopsin null mutations do not show clinical evidence of RP, although they may present with electrophysiological abnormalities[42; 81; 88; 89]. In addition, transgenic animals overexpressing the P23H allele on a wild-type rhodopsin background present with retinal degeneration. Overexpression of wild-type rhodopsin in P23H transgenic mice slows the progression of retinal degeneration, indirectly suggesting a dominant-negative effect of the mutated allele[90]. Several independent observations point to a gain-of-function effect exerted by the P23H mutation. Its overexpression in

cultured cells results in its accumulation in the endoplasmic reticulum (ER) as a consequence of the incorrect folding of the mutated protein[91]. In the ER, misfolded P23H aggregates and overwhelms the proteasome system, leading to cell death[91; 92].

Mutations resulting in changes in the C-terminal domain of rhodopsin result in some of the more severe forms of the disease, with total blindness occurring in early adulthood. The rhodopsin C-terminal sequence is recognized by specific factors in the trans-Golgi network. Mutations resulting in changes in this region produce RP due to formation of abnormal post-Golgi membranes and from the aberrant subcellular localization of rhodopsin[93-95]. Among the class I mutants, those affecting the proline in position 347 near the C-terminus seems to be extremely deleterious. This residue is conserved among all known visual pigments. Six different missense mutations affecting this residue have been identified among patients with RP, indicating that proline-347 mediates some vital function of rhodopsin. Proline-347 mutants are of particular interest, since no functional abnormalities of the mutant proteins have been observed in *in vitro* studies, yet they tend to cause a more severe form of RP than that found in patients with adRP due to other mutations[96]. A transgenic mouse carrying a human P347S allele was generated to investigate the early pathogenic events by which this rhodopsin mutant leads to photoreceptor cells death[94]. In the pre-degenerate or early degenerating retinae of these mice there was no gross perturbation of phototransduction, as monitored by ERGs, and no accumulation of mutant rhodopsin. However, the mutant retinae exhibited a distinct morphological phenotype (extracellular accumulation of small rhodopsin-containing vesicles in the vicinity of basal outer segments and apical inner segments) which suggests that the C-terminus of rhodopsin might have a role

in rhodopsin transport to the outer segments[95]. Photoreceptor cell death in the P347S mice can be thus a consequence of this primary defect.

To further elucidate the mechanisms of photoreceptor cells death induced by rhodopsin mutations, also transgenic porcine model for the rhodopsin P347L and P347S mutations have been produced and show a disease phenotype similar to that found in humans[86; 97; 98].

3. ADENO-ASSOCIATED VIRUS (AAV) AS A TOOL FOR *IN VIVO* GENE TRANSFER

The success of gene therapy approach is based on an appropriate amount of a therapeutic gene delivered into the target tissue without substantial toxicity. Each viral vector system is characterized by an inherent set of properties that affect its suitability for specific gene therapy applications. For some disorders, long-term expression from a relatively small proportion of cells would be sufficient (for example, genetic disorders), whereas other pathologies might require high, but transient, gene expression.

To date, three types of viruses have mainly proven useful for retinal transgene delivery. They are Adeno-Associated Virus (AAV), Lentivirus and Adenovirus[53; 99]. These viruses are genetically modified to eliminate the inherent toxicity caused by their ability to replicate and disseminate[51]. Among these vector systems AAV-based vectors are very promising. AAVs are not associated with human diseases and possess a number of properties that make them particularly suitable for clinical gene therapy, including the efficiency to transfer genetic material to a number of dividing and not dividing cells and a natural propensity to persist in human cells. In addition, the development of AAV vectors for gene therapy so far has shown an excellent safety record with data accumulated from thousands of animal studies and hundreds of human patients[51; 100].

3.1 General overview on AAV

The adeno-associated virus (AAV) is a small (20-25 nm in diameter), non-enveloped, icosahedric *Dependovirus* belonging to the *Parvoviridae* family[101]. These viruses are non pathogens for humans and possess a linear, single-

stranded DNA genome that can replicate in the presence of different helper viruses such as adenovirus, herpesvirus or papillomavirus[102]. AAV was originally isolated as a contaminant of adenoviral cultures and thus given the name adeno-associated virus. It is native of humans and non-human primates (NHP) and exists in nature in more than 100 distinct variants, including both those defined serologically as serotypes and those defined by DNA sequence as genomovars[103; 104]. The AAV genome (4.7 Kb) consists of two open reading frames: i) *rep*, which codes for a family of multifunctional non-structural proteins that are involved in viral genome replication, transcriptional control, integration and encapsidation of rAAV genomes into preformed capsids[105-109] and ii) *cap* encoding for the three structural proteins VP1, VP2 and VP3[110; 111]. *rep* and *cap* are flanked by viral T shaped palindromic elements, the inverted terminal repeats (ITRs) which are 145 nucleotides in length[102]. *In vitro* experiments demonstrated that, in the absence of an helper virus, AAV establishes latency by integrating in a site-specific manner in the human chromosome 19q13.3-qter (in a site called AAVS1)[112] through an interaction between the ITRs and the AAVS1 locus mediated by the rep proteins[113]. Despite the presence of a preferential integration site, the status of AAV genomes from infected cells has been shown to be mainly episomal[114; 115].

3.2 Advantages and limitations of recombinant AAV (rAAV)

The conversion of an AAV isolate into recombinant vector (rAAV) to be used in gene therapy is obtained by exchanging the viral coding sequence between the ITRs with the therapeutic gene[116]. To produce rAAV, the rep and cap functions as well as the helper genes needed are provided in *trans*[117]. In the absence of rep, rAAV loses its site-specific integration ability[118]. The most commonly used

strategy to produce rAAV vectors to be used in gene therapy is based on the co-transfection into permissive cells (usually human embryonic kidney 293 cells) of three separate plasmids[116; 117]: i) a plasmid containing the viral ITRs flanking the therapeutic gene cassette; ii) a packaging plasmid encoding for the rep and cap proteins; iii) the helper plasmid encoding for the essential adenoviral helper genes (**Fig. 9**)[116; 117]. The versatility of the rAAV vectors is that

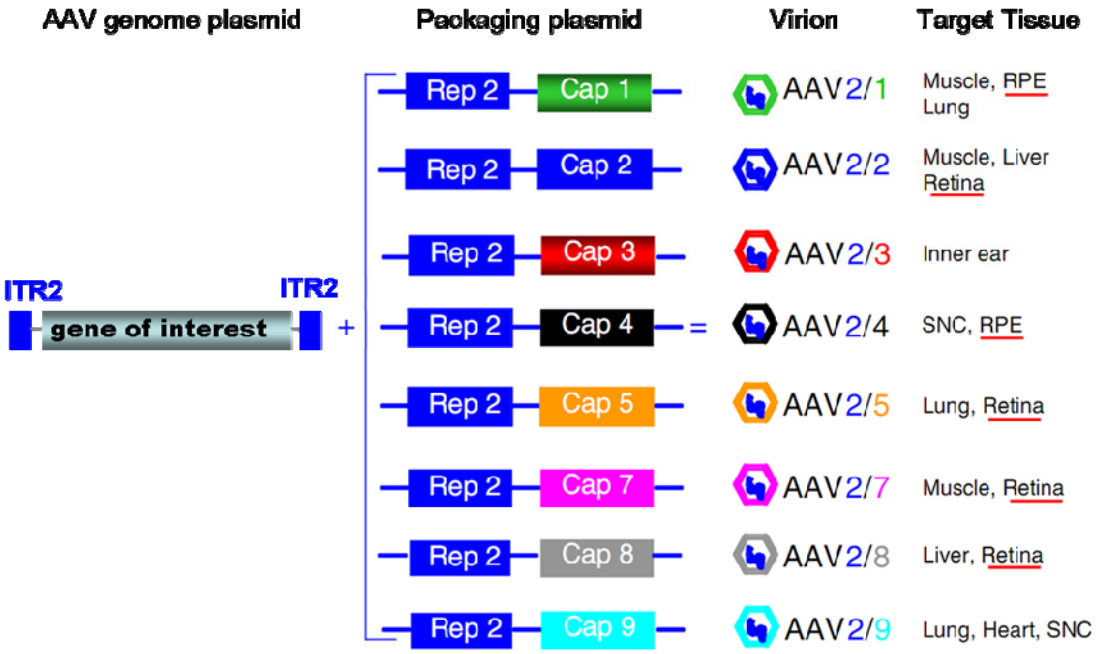

Figure 9. Schematic representation of rAAV vector production. The hybrid virions containing the genome of one serotype (AAV2) and a different capsid (AAV2/1 to AAV2/n) have a different tropism dependent on the capsid proteins.

the cap genes in the packaging plasmid can be interchanged between different AAV serotypes isolated (from AAV1 to AAVn) and cloned allowing the assembly of hybrid rAAV with the vector genome (encoding the therapeutic gene) from one serotype (the most studied and commonly used is for example the genome from AAV2) and the capsid from a different AAV serotype[119; 120]. These hybrid vectors are named rAAVx/y where x indicates the serotypes of origin of the genome and the y is the capsid[119]. Since capsid proteins are the main determinants of rAAV tropism and transduction characteristics (intensity and

onset of gene expression)[121; 122], vectors with different capsids have different abilities to transduce target cells *in vivo* (**Fig. 9**). This can be partly explained by the presence of specific receptors for AAV serotypes on the membrane of target cells. For example in the case of rAAV2/2, capsid proteins interact with a membrane receptor complex including heparin sulphate proteoglycans, fibroblast growth factor receptor 1 and integrin[123-125], while rAAV2/5 interacts with O-linked sialic acid and platelet derived growth factor receptor[126; 127]. The absence of the receptor complex for rAAV2/2 on the luminal surface of airways epithelia and the presence of O-linked sialic acid explain the ability of rAAV2/5, but not of rAAV2/2, to transduce the lung *in vivo*[128; 129]. It's highly likely that post-entry events can be additionally influenced by different AAV viral capsids.

Compared to other viral vectors, rAAV induces little or no innate immunity, probably due to the lack of viral sequence other than the ITRs[130]. In addition, rAAV generally elicits a reduced cellular immuno-response against the transgene product, probably due to the inability of rAAV vectors to efficiently transduce or activate mature antigen presenting cells (APCs)[131]. Both the humoral and cell-mediated response to the delivered transgene depend on a number of variables including the nature of the transgene itself, the promoter used, the route and site of administration, the vector dose and the host factors[132-134]. The majority of these variables can be suitably modified. Humoral and, more recently, cell-mediated immune responses to the rAAV virion capsid have been consistently detected in animals and humans following rAAV vector delivery[103; 104; 133; 135-137]. The presence of neutralizing antibodies and cell-mediated immunity against capsid proteins has been shown to prevent or greatly reduce the success of vector re-administration and to limit the duration of transgene expression[133; 135-139]. Several studies have suggested that evasion

of the immune response against the rAAV capsid can be obtained by using different serotypes by capsid modification or by immunosuppression[132; 134; 136]. One of the major drawback of rAAV vectors as tools for *in vivo* gene transfer is their relatively small packaging capacity (4.7 Kb) which limits the possibility to develop therapies for diseases caused by mutations in large genes such as Duchenne muscular dystrophy, cystis fibrosis, Stargardt's disease and others[140; 141]. Various strategies have been adopted to overcome this limitation taking advantage of the propensity of AAV genomes to form head-to-tail concatamers through intermolecular recombination[108; 142-146].

Despite this limitation, the absence of human diseases associated with their infection, the low toxicity and immunogenicity, the ability to transduce both dividing and non-dividing cells and the possibility to use a specific serotype to transduce a target tissue make rAAV an ideal candidate vehicle for gene therapy applications *in vivo*.

3.3 rAAV as a gene transfer vehicle for the retina

rAAV are promising vectors for gene therapy in the retina since they can transduce dividing and non-dividing cells[102], mediate efficient and prolonged transgene expression[147; 148] and are able to transduce the retina with a different cell tropism and efficiency[119]. To date, rAAV vectors have been used to improve the efficiency of transduction in different retinal cell layers[121; 149; 150] which are affected in many inherited and non-inherited blinding diseases[28]. Subretinal injections of rAAV2/2, rAAV2/5, rAAV2/7 and rAAV2/8 in rodents result in efficient transduction of photoreceptor cells and RPE cells[121; 151]. rAAV2/5-mediated transduction peaks 5 days post-treatment, when rAAV2/2 begins to express. Another characteristic of rAAV2/5 is that it is able to transduce a

considerably higher number of photoreceptors than rAAV2/2 (with a ratio of 400:1, 15 weeks after transduction) reaching a number of genomic copies per eye 20 folds higher than rAAV2/2[121; 150]. Interestingly, rAAV2/7 and rAAV2/8 mediated six to eight fold higher levels of in vivo photoreceptor transduction than rAAV2/5[151]. Many of the features of rAAV-mediated retinal transduction in rodents have been validated in feline, canine and non-human primates (NHP) models[55; 56; 147; 152-155]. In NHP, rAAV2/2 efficiently targets rod cells and RPE and is not able to transduce cones, whereas rAAV2/5 appears to be more efficient than rAAV2/2 in transducing rod photoreceptors[147; 154]. The RPE has been efficiently transduced by subretinal injections of rAAV2/4 that seems exclusive for this cell type, in which it allows a stable transgene expression in rodents, canines and NHP[150; 156]. rAAV2/1 and rAAV2/6 exhibit a higher RPE-transduction specificity and efficiency and a faster expression than rAAV2/2[121; 150]. rAAV2/3 poorly transduces the retina following subretinal administration, possibly due to the absence of a specific receptor or co-receptor essential for capsid interaction with cellular membrane[156]. rAAV2/2 is the only rAAV vector able to efficiently transduce retinal ganglion cells, the trabecular meshwork and different cells of the inner nuclear layer, upon intravitreal injections[150; 157]. rAAV vectors can efficiently transduce neuroprogenital retinal cells, with transduction characteristics depending on the time of administration. For example, subretinal injection of rAAV2/1 at embryonic stage 14.5 (E14.5) results in expression of the transgene in various cell types while if given at post-natal day 0 (P0), the transgene expression is confined to the RPE and photoreceptors[158]. Similarly, fetal retina is barely transduced by rAAV2/2 while the same vector can transduce various retinal cell types if administered soon after birth; finally while subretinal fetal administration of rAAV2/5 results in transduction of cone

photoreceptors, amacrine and ganglion cells, when given at birth it transduces both cones and rods as well as Mueller cells[158].

In addition, to restrict transgene expression to particular cell types in the retina, the use of tissue-specific promoters can be exploited. Among them, promoter fragments as well as *cis*-acting elements from the *RPE65* or *VMD2* genes have been coupled to the proper rAAV serotype to target RPE[55; 159]. In 1997 Flannery and colleagues[148] used the proximal promoter region of the mouse rod opsin promoter located within -385 to +36 (RPPR) to restrict rAAV2/2 expression specifically to rat photoreceptors. Moreover, Glushakova and collaborators[160] have shown that this promoter is photoreceptor specific but not rod-specific: subretinal injections in rats of rAAV2/5 expressing RPPR-driven eGFP resulted in both rods and cones transduction, suggesting that new insights are necessary in order to achieve specific transgene expression in photoreceptors.

Thus, in order to think to the development of a rAAV vector as gene therapy vehicle to delivery transgene product to a target cell, it is extremely important to consider which capsid to use, the promoter element and the route of administration of the vector. To date, among the vectors tested, the best combination in terms of transduction efficiency and transgene expression levels into photoreceptor cells can be obtained using a rAAV2/8 and the human rhodopsin promoter. These date rise from the experiments performed by Allocca and colleagues, a study in which I took part[151].

4. ZINC-FINGER-BASED ARTIFICIAL TRANSCRIPTION FACTORS (ZF-ATFS)

A variety of biological processes including development, differentiation and diseases are regulated through gene expression which is mainly modulated by transcription factors. These proteins are able to bind to specific sites on the promoter region of target genes thus controlling their expression. This in principle suggests that the generation of "artificial" transcription factors (ATFs) tailored to the promoter of a given gene enable the transcriptional control of the target thus impacting on the downstream process. This idea paves the way for the development of novel gene therapy approaches aimed at controlling the expression of a disease gene.

Transcription factors are protein mainly composed by three elements: i) a DNA binding domain (DBD); ii) an effector domain to control the expression of the target; iii) a nuclear localization signal (NLS) to deliver the transcription factor to the nucleus where the transcription mechanism occurs. Many proteins are able to bind DNA but zinc finger proteins (ZFPs) have been chosen for the generation of artificial transcription factors on the basis of their plasticity, modularity and because they bind the DNA as monomer. This is a very important characteristic since usually promoter regions have no palindromic sequences. One of the most common zinc finger-based DBD in eukaryotes is the Cys_2His_2 type zinc finger domain which comprises multiple repeats of approximately 30 amino acids with a simple $\beta\beta\alpha$ fold stabilized by hydrophobic interactions and the chelation of a single zinc ion (**Fig. 10**). It was first discovered in the transcription factor TFIIIA of Xenopus laevis in 1985[161]. Classically, one unit of the Cys_2His_2 zinc-finger domain was thought to contact 3-bp of the DNA target. Pabo's group reported the X-ray crystal structure of the DNA complex with the endogenous transcription factor Zif268 at 2.1 A

resolution[162]. In the complex, amino acids at positions −1 and 6 of the α-helical region of the zinc-finger domain (position 1 is the starting amino acid in the α-helix) in the 1st and 3rd fingers contacted the 1st and 3rd bases, and amino acids at positions −1 and 3 in the 2nd finger contacted the 1st and 2nd bases. However, no other amino acid–base contact was observed at that resolution. After Pabo's reports, Berg's group reported important structural information on DNA recognition by ZFPs. They revealed the crystal structure of the DNA complex of their designed ZFP at 2.2 A resolution[163]. The ZFP comprising a consensus finger framework[164] was selected from a family of three-fingers proteins that had been prepared and characterized with regard to their DNA-binding specificities[165-168]. The X-ray crystal structural analysis of the DNA complex revealed the following important features: i) each zinc-finger domain recognizes an overlapping 4-bp DNA sequence, where the last base pair of each 4-bp target is the first base pair of the next 4-bp target (**Fig. 11**); ii) in all three fingers of the protein, amino acids at specific positions

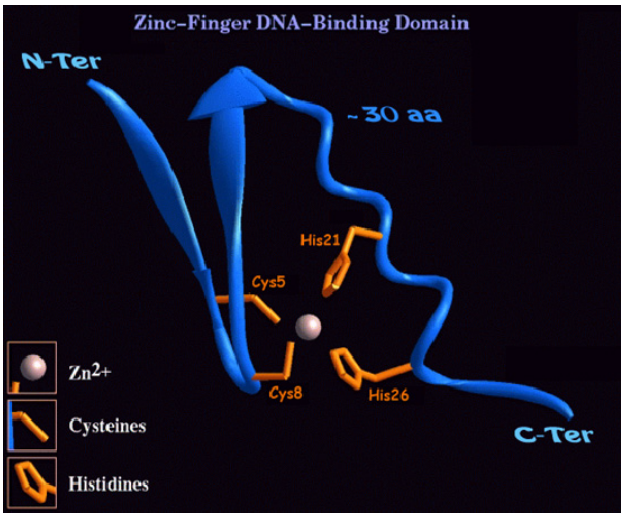

Figure 10. Schematic representation of typical Cys_2His_2-type zinc-finger motif displaying ββα fold.

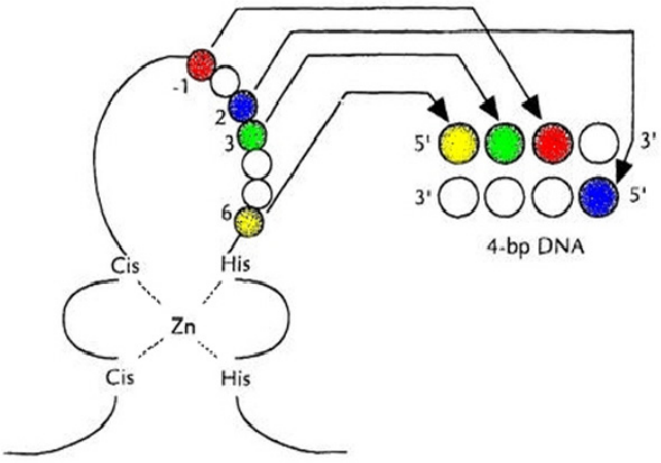

Figure 11. Schematic view of the binding. Defined amino acids contact DNA bases at specific positions.

contact DNA bases at specific positions in a regular fashion. Namely, amino acids at positions −1, 2, 3, and 6 contact the 3rd, 4th, 2nd, and 1st bases of the overlapping 4-bp DNA targets, respectively (only the 4th base in the antisense strand; **Fig. 11**). Whether a one-finger domain recognizes a 3- or 4-bp DNA remains unclear and many papers and reviews still report that one zinc-finger domain recognizes 3- or 4-bp.

In 1992, Berg's group reported the first example of ZFPs with altered binding specificities[169]. They mutagenized the amino acid at position 3 of the 2nd finger of the protein Sp1 based on a database of zinc-finger sequences available at the time and presented a first partial “recognition code” of GNG and GNT triplets. After these pioneering studies, several groups reported the generations of ZFPs with new DNA-binding specificities making the generation of ZFP-based ATFs practical.

4.1 Generation of ZF-ATFs

The most practical way to obtain effective ATFs is to screen a panel of ATFs that individually target different sequences in the promoter region of the gene of interest in a high-throughput manner[170]. As a consequence, the first step in the generation of a ZF-ATFs is the choice of the endogenous target gene to modulate. To allow the ATFs to bind the desired target, the accessibility of genomic DNA in chromatin is crucial[171]. Moreover, an important aspect is that the ATF would be targeted to a unique site in the genome and this can be achieved using long DNA target sequences. Assuming that a single ZF module binds 3-bp, it is therefore theoretically necessary to recognize >16-bp DNA for specific recognition of a single DNA target site in a human cell considering that the genome size is 3×10^9 bp. As a consequence, at least 6 ZF modules should

be joined together in order to recognize a DNA target site of 18-bp. To date, a recognition code table has been generated which allows to recognize 49 of the 64 DNA triplets[172-176] and the modular assembly of the DBD can be also facilitated through the use of the Zinc Finger Tools website based on Barbas' modules (http://www.zincfingertool.org)[177]. Moreover, two-finger building blocks made by Sangamo Bioscience are commercially available via Sigma-Aldrich[76]. Once that a panel of ZF-based DBDs targeted to the desired gene are obtained, they could be expressed and purified in order to examine their binding properties determining the apparent dissociation constant (K_d) through techniques such as Electromobility Shift Assay (EMSA). The best or better DBDs are fused to a nuclear localization signal (NLS), to an epitope tag such as FLAG, c-myc or HA to monitor ATF expression, and to an effector domain to generate ATFs. The most frequently used transcriptional activator is the VP-64 domain[178] while the most popular transcriptional repressor is a Kruppel-associated box (KRAB) domain of KOX1[179]. Finally, the resulting ATFs are then cloned into mammalian expression plasmids or viral vectors and their functionality is investigated by transient reporter assays or more ideally by analyzing the mRNA levels of target genes by Northern blotting or quantitative real-time PCR. The efficacy and the specificity of the ATFs can be evaluated by microarray analysis, DNase I footprinting assay or chromatin immunoprecipitation (ChIP).

4.2 ZF-ATFs applications

The first study of gene regulation by altered transcription factors in living cells (yeast) was reported in 1992[180]. Since then, several groups spent their effort in order to develop this technology to achieve the transcriptional control at will of a

given gene with therapeutic purposes. The first 6-finger ATF was reported in 1998[178] to modulate the expression of the ErbB-2 which is overexpressed in a high percentage of human adenocarcinomas. A 6-finger ATF targeted to the promoter region of the ErbB-2 gene was generated and demonstrated a successful and specific up- and down-regulation of the target gene *in vitro*. The first example of ATF-mediated gene regulation *in vivo* was reported in 2002[181] in a study sponsored by Sangamo Bioscience, a biotechnology company worldwide leader in the design and development of engineered zinc finger DNA-binding proteins (ZFPs) for gene regulation and gene modification). They designed ZFPs to regulate the endogenous gene encoding vascular endothelial growth factor-A (*VEGF-A*) which is an endothelial cell-specific mitogen that is a key inducer of new blood vessel growth, both during embryogenesis and in later processes such as wound healing. *VEGF-A* levels are dramatically increased by hypoxia, triggering angiogenesis and microvascular permeability. Therefore, both activation and repression of *VEGF-A* are attractive therapeutic approaches. They showed that expression of these new ZFPs *in vivo* led to induced expression of the protein VEGF-A, stimulation of angiogenesis and acceleration of experimental wound healing[181] thus establishing for the first time the feasibility and potential utility of this approach as new tool for gene therapy. Two years ago, ZF-based ATFs entered phase II clinical trials[76].

AIMS

The aim of the project of my Ph.D. project was the development of a novel gene therapy approach for the treatment of inherited retinal degenerations due to gain of function mutations. We developed a strategy based on zinc finger technology to repress the expression of the rhodopsin, the gene most commonly associated with autosomal dominant *Retinitis Pigmentosa*, a severe inherited retinal degeneration with a prevalence of 1:3500 worldwide. The possibility to silence transcriptionally rhodopsin would allow to prevent the pathological consequences of the expression of the mutated allele, thus halting the patho-physiological cascade of events that lead to retinal degeneration. The strategy employed is mutational-independent because transcriptional silencing does not discriminate between the mutated and wild-type alleles. Thereby, based on this characteristic feature, in principle any gain of function mutation can be treatable; however, since also the wild type allele is silenced, a combined repression and replacement system will be developed in the future.
The specific aims of my project were the following:

1. Generation and characterization of engineered zinc finger transcriptional repressors targeted to the human rhodopsin promoter.
To control the expression of the rhodopsin gene we generated a series of polydactyl ZF-based transcription factors fused either to an activator (VP64) or to a repressor (KRAB) in order to modulate the expression of the target gene.

2. Assessment of the efficacy of RHO transcriptional repression *in vivo* and impact on the disease progression in an adRP mouse model.

Based on *in vitro* data in aim 1 I selected a ZFR and enclosed it in an AAV2/8 vector for photoreceptor delivery in a mouse model of adRP expressing a mutated human rhodopsin (P347S). This animal model develops retinal degeneration with fast progression. The levels and specificity of transcriptional repression were measured. In vivo expression of the ZFR in the diseased photoreceptor resulted in the specific downregulation of the transgenic human rhodopsin leaving unaltered the expression levels of the endogenous murine allele. This in turn ameliorated the retinal phenotype functionally and morphologically as measured by electroretinograms and histological the progression of the disease.

Materials and Methods

MATERIALS AND METHODS

Rational design of the artificial Zinc-Finger based Transcription Factors (ZF-TFs)

The protein sequences of the DNA Binding Domains (DBDs) targeted to the 10 different target sites (see **Table 2** in Results section) were obtained using the web-based Zinc Finger Tools (http://www.zincfingertools.org). Each DBD was composed of two arms each recognizing a 9-bp half target site and fused together through a linker sequence in order to overcome the gap sequence. We used two different linkers according to the length of the gap: for gaps longer than 3-bp (target sites from 1 to 4) we used a complex structured linker, as reported by Moore[186] (GRSSVESACVTSVLVALLPATSAPTQVSG) while for longer gaps (target sites from 5 to 10) we used a flexible linker, as described by Rebar[181] (QNKKGGSGDGKKKQHA). We optimized the corresponding DNA sequences to facilitate the subsequent cloning steps and purchased them as transgene in the pLS-standard vector at “TOP Gene Technologies”, Quebec, Canada. After the *in vitro* selection steps, we purchased an additional DBD with the fingers of the DBD number 6 shuffled (from 1.2.3-4.5.6 to 5.1.6-3.4.2) to be used as a negative control for a total of 11 DBDs. All the DBDs were flanked by a ClaI and an XbaI site at the 5’ and 3’ end, respectively.

DBD number 1 was purchased as a complete protein fused at the N-Ter with the repressor domain KRAB (Krüppel-associated box, DAKSLTAWSRTLVTFKDVFVDFTREEWKLLDTAQQILYRNVMLENYKNLVSLG YQLTKPDVILRLEKGEEPWLVEREIHQETHPDSETAFEIKSSV) and the nuclear localization signal (NLS, PKKKRKV) from SV40 large T antigen and fused at the C-Ter with the HA-tag (YPYDVPDYAS). Also DBD number 2 was

purchased as a complete protein but fused at the C-Ter with the NLS, the activator domain VP64 (tetrameric repeat of herpes simplex VP16's minimal activation domain, GRADALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLDMLGSDALDDFDLD MLIN) and the HA-tag. In the two proteins, the DBDs can be exchanged through a double digestion with the restriction endonucleases ClaI and XbaI.

The 11 different transcription activators (ZF-As) and repressors (ZF-Rs) were inserted in the pAAV2.1-CMV-EGFP NotI and HindIII sites, downstream of the CMV (Cytomegalovirus) promoter by removing the EGFP coding sequence for transfection experiments. For AAV preparations, ZF-R6 was inserted in the pAAV2.1-RK-EGFP NotI and HindIII sites, downstream of the RK (Rhodopsin Kinase) promoter by removing the EGFP coding sequence. For retrovirus preparations used in Retinal Stem Cell experiments, coding sequences of ZF-R2, ZF-R6 and ZF-R6 shuffled were cloned in LXSN plasmid EcoRI and HindIII sites downstream of the CMV promoter.

Reporter Constructs

Luciferase reporter plasmids were generated from the pGL3-Basic vector (Promega, Madison, WI) by cloning the proximal upstream regions of the human or murine rhodopsin gene. The human rhodopsin promoter (*hRHO*-P) extending from -330 to +92 relative to the transcription start site was PCR amplified using the FastStart High Fidelity PCR System (Roche) from a human genomic DNA sample using the following primers: hRHO-P.Nhe forward 5'-TTTTGCTAGCTGTCCAGAGGACATAGCAC-3' and hRHO-P.EcoRV reverse 5-AAAGATATCGGCTGTGGCCCTTGTGGCTGA-3'. For the murine rhodopsin promoter (mRho-P) extending from -404 to +96 we used a murine genomic

DNA sample and the following primers: mRho-P.Nhe forward 5'-TTTTGCTAGCTCAGATCTGTCAAGTGAGCC-3' and mRho-P.HindIII reverse 5'-AAAAAGCTTGGCTGCGGCTCTCGAGGCTG-3'. The two fragments were inserted within the NheI/SmaI or NheI/HindIII sites of pGL3-basic vector obtaining pGL3-hRHO-P-Luciferase or pGL3-mRho-P-Luciferase, respectively.

Selection of the functional ZF-TFs in human cells by transient transfection

HEK293 cells were plated in 6-well plates at a density of 400.000 cells/well. Twenty-four hours later, the cells were co-transfected using FUGENE 6 (Roche, Basel, Switzerland) following the manufacturer's instructions. Transfection cocktails included 200ng of reporter plasmid (or pGL3basic as a negative control), 300 ng of ZF-TF, 300 ng of a plasmid encoding for the murine CRX (alone as a positive control in transactivation assay or in combination with the ZF-Rs for the repression assays) and 10 ng of Renilla Luciferase reporter plasmid control (Promega) to normalize for transfection efficiency. The amount of DNA was kept constant by adding pAAV2.1-TBG-EGFP to a total of 1 μg. Cells were harvested 48 h after transfection in 1x PLB Lysis Buffer (Promega). Firefly and Renilla Luciferases activities were measured in a GloMax™ luminometer (Promega) using Dual-Luciferase Reporter Assay System (Promega).

Electromobility Shift Assay (EMSA)

HEK293 cells were transfected with a plasmid encoding the ZF-R2, ZF-R6 or ZF-R6 shuffled using FUGENE 6 as described above. After 48 h, cells were harvested and nuclear extracts were prepared using NE-PER Nuclear and Cytoplasmic extraction kit (Pierce) according to the manufacturer's instructions.

The presence of the proteins in the nuclear fractions was assessed by Western Blotting analysis using as primary antibody a rabbit anti-HA (1:100; SIGMA, Milan, Italy). Electromobility Shift Assay was performed using Lightshift Chemiluminescent EMSA kit (Pierce) following the manufacturer's instructions. In brief, nuclear extracts (ZF-R2, ZF-R6 or ZF-R6 shuffled) were incubated with equal amounts (20 fmol) of the specific byotinilated double stranded probe (5'-TGTGGGGGTTAGAGGGTCTACGACTA-3') to asses the specific shift for 20 min at room temperature. The competition control was performed adding a molar excess of the unlabeled probe to the mix. The sequence of the unspecific was the following: 5'-TGCGGCGGCTAAAGGCTCGACAACGA-3'. The supershift was obtained adding a rabbit anti-HA (1:100; SIGMA, Milan, Italy) primary antibody at the mix.

AAV vector production and purification

AAV vectors were produced by the TIGEM AAV Vector Core using pAAV2.1-RK-ZF-R6 and pAAV2.1-CMV-EGFP. Recombinant AAV8 viruses were produced by triple transfection of HEK293 cells followed by CsCl2 purification of the vectors, as previously described[121]. For each viral preparation, physical titers (GC/ml) were determined by both PCR quantification using TaqMan (Perkin–Elmer, Life and Analytical Sciences, Inc.) and dot-blot analysis.

RNA preparation and measurement of rhodopsin transcript levels by Real Time PCR

RNAs from tissues or cells were isolated using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol. cDNA were amplified from 500 ng of isolated RNA using the SuperScript™ III First-Strand Synthesis (Invitrogen) as

indicated in the attached instructions. Transcripts levels of human and murine rhodopsin were measured by Real Time PCR using the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City,CA) and the following primers: hRho_forward 5'…CCGGCTGGTCCAGGTACAT….3' and hRho_reverse 5'…TTGTTGACCTCCGGCTTGAG…3'; mRho_forward 5'…TGTTGAACAAGCAGTTCCGGA...3' and mRho_reverse 5'…TCGTCATCTCCCAGTGGATTC...3'. The PCRs with cDNA were carried in 25 μl total volume using 12.5 μl SYBR Green Master Mix (Applied Biosystems) and 400 nM primers under the following conditions: preheating, 50°C for 2 min and 95°C for 10 min; cycling, 40 cycles of 95°C for 15 s and 60°C for 1 min. All the reactions were standardized against murine GAPDH by using the following primers: mGAPDH_forward 5'…CTTCACCACCATGGAGAAGGC…3' and mGAPDH_reverse 5'…CTCATGACCACAGTCCATGCC…3'. To quantify expression levels of photoreceptor specific genes (*Peripherin*, *PDE6β* and *Cnga*) we used the following primers: for *Peripherin*: mPeriph.forward 5'-CGGGACTGGTTCGAGATTC-3' and mPeriph.reverse 5'-ATCCACGTTGCTCTTGATGC-3'; for *PDE6β*: PDE6β.forward 5'-TGAAGATGAAGATGTTTTCACG-3' and PDE6β.reverse 5'-CTCTGCGTGTCTCACAGTTG-3'; for *Cnga*: Cnga.forward 5'-ACTCGTACAAAAGGCGAGGAC-3' and Cnga.reverse 5'-CTTTGTTGCTGCTGTTGTTGAC-3'. Each sample was analyzed in triplicate in two independent experiments.

Retinal stem cells culture and analysis

All procedures on mice (including their euthanasia) were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and with institutional guidelines for animal research.

Retinal stem cells were isolated from adult P347S mice and were incubated for 5 days until floating spheres formed in serum free medium (0.6% glucose and N2 hormone mix in DMEM-F12) containing 20 ng/ml basic FGF supplemented with 2 µg/ml heparin (Sigma, Milan, Italy), as previously described. Retinal floating spheres were allowed to differentiate on eight well glass slides that were coated with extracellular matrix (ECM, Sigma) in DMEM-F12, supplemented with 1% FBS (Gibco, San Giuliano Milanese, Italy). Analysis was performed 12 days after induction of differentiation. Apoptotic nuclei were detected by TdT-dUTP terminal nick-end labeling kit (TUNEL, Roche Diagnostics, Monza, Italy) according to the manufacturer's protocol. For immunofluorescence staining, cells were washed once with PBS and then fixed for 10 min in PBS containing 4% PFA. After fixation, cells were washed and incubated 5 min in sodium citrate buffer 0.1M containing 0.2 % Triton X100. Blocking solution containing 3% bovine serum albumin (Sigma, Milan, Italy) was applied for 1 h. Primary antibodies were diluted in PBS and incubated 90 min at RT. The primary antibodies used were: rabbit anti-HA (1:100; SIGMA, Milan, Italy) and mouse anti rhodopsin 1D4 (1:400; SIGMA, Milan, Italy). Cells were then washed in PBS and incubated with secondary antibodies: 1:1000 Alexa Fluor® 568 anti-rabbit, 1:1000 Alexa Fluor® 568 anti-mouse (Molecular Probes), and 1:500 Cy5 anti-rabbit (Jackson Immunoresearch, West Grove, PA, USA). Slides were coverslipped with Vectashield (Vector laboratories,

Burlingame, CA, USA) and photographed using either an Axioplan microscope (Zeiss) or a Leica Laser Confocal Microscope System.

To allow ectopic expression in differentiated retinal cells, ZF-R2, ZF-R6 and ZF-R6 shuffled were cloned in Moloney murine leukemia virus-based vector LXSN as described above (see first paragraph of this section). Retinal cell cultures were transduced at day 7 post explant with retroviruses and cells were analyzed 4 days later.

Animal model, vector administration, and tissue collection

All procedures were performed in accordance with institutional guidelines for animal research. P347S +/+ animals for breeding were kindly provided by Dr. G. Jane Farrar (Smurfit Institute of Genetics, Trinity College Dublin, Dublin, Ireland) and were bred in the animal facility of the Biotechnology Center of the Cardarelli Hospital (Naples, Italy) with C57BL/6 mice (Charles Rivers Laboratories, Calco, Italy) to obtain P347S +/- mice. At P4, animals were anesthetized on ice and subretinally injected in the right eye with 1 μl AAV2/8-RHOK-ZF-R6 vector ($6.3*10^{12}$ GC/ml) and 1 μl of AAV2/1-CMV-EGFP ($6.0*10^{12}$ GC/ml) in mix whereas the contralateral eye received, as control, the same dose of AAV2/1-CMV-EGFP and AAV2/8-RHOK-EGFP vectors in mix. Mice were sacrificed at postnatal day 18 or at 1 month of age (P18 or P30). For morphological studies, eyecups were harvested, fixed by immersion in 4% paraformaldehyde and then embedded in OCT (kaltek). For RNA extractions, the isolated retinae were removed and snap-frozen in liquid nitrogen and treated as described in the above RNA preparation section.

Histological analysis

For each eye, 150 to 200 serial sections (5-μm thick) were cut along the horizontal plane; the sections were progressively distributed on 10 glass slides so that each slide contained 15 to 20 sections representative of the whole eye at different levels. Slides were coverslipped with Vectashield containing DAPI (4',6-diamidino-2-phenylindole; Vector laboratories, Burlingame, CA, USA) to stain cells nuclei and retinal histology was analyzed an Axioplan microscope (Zeiss). To quantify photoreceptor rescue, the number of nuclei in the outer nuclear layer (ONL) of each eye was counted. For each section, the number of nuclei in the ONL was separately counted on the nasal, central, and temporal sides. The nasal, temporal, and central counts of each section were independently averaged, therefore obtaining a number that was the average of the three sides for each eye. The counts from each group (treated and untreated) were then averaged and standard errors were calculated.

Electroretinogram measurements

ERGs were performed at P30, P60 and P90. Briefly, animals were adapted to the dark for 3 h and then anesthetized and accommodated in a stereotaxic apparatus under dim red light. ERGs were evoked by flashes of different light intensities ranging from 10^{-4} to 20 cd.s/m^2 generated through a Ganzfeld stimulator (Lace). To minimize the noise, three different responses evoked by light were averaged for each luminance step. The electrophysiological signals were recorded by gold plate electrodes inserted under the lower eyelids. Electrodes in each eye consisted of a needle electrode inserted subcutaneously at the level of the corresponding frontal region. The different electrodes were connected to a two-channel amplifier. Amplitudes of a-and b-waves were

plotted as a function of increasing light intensities. After completion of responses obtained under dark-adapted conditions the recording session continued with the aim to dissect the cone pathway mediating the light response. To this end the ERG in response to light of 20 cd.s/m^2 was recorded in the presence of a continuous background light (background light set at 50 cd/m^2). The amplitude of the b-wave for each eye was plotted as a function of luminance under scotopic and photopic conditions. For each group the mean b-wave amplitude was plotted.

Statistical analysis

Data are expressed as mean ± s.e.m. as indicated and compared by two-tailed Student's t-tests, two-sample equal variance.

Results

RESULTS

1. GENERATION AND CHARACTERIZATION OF ENGINEERED ZINC FINGER TRANSCRIPTION REPRESSORS TARGETED TO THE HUMAN RHODOPSIN PROMOTER

1.1 Design and generation of Zinc Finger-based transcription factors to control rhodopsin gene expression

To control the rhodopsin gene expression we designed Zinc Finger-based Artificial Transcription Factors (ZF-ATFs) targeted to the human rhodopsin promoter (hRHO-P). We first scanned the proximal hRHO-P to identify amenable target regions applying the following criteria: (i) exclusivity of the 18-bp DNA sequences in the human genome, (ii) putative accessibility to the chromatin environment (in proximity of known endogenous transcription factors binding sites)[182], (iii) absence of annotated polymorphisms and (iv) divergence between the human and murine rhodopsin promoter target sequences. We selected 10 different continuous or discontinuous 18-bp target sites within the proximal region of the hRHO-P (from -90 to -32, relative to the transcription

Figure 12. DNA sequence of the human rhodopsin proximal promoter region. lines represent the relative positions of DNA sequences targeted by the 10 different DBDs. Boxes highlight putative binding sites for known endogenous regulators of hRHO

start site; **Fig. 12** and **Table 2**) that fulfilled the 4 criteria described above. To obtain a DNA Binding Domain (DBD) tailored to a unique target sequence in

mammalian genomic DNA (3,0x10^9 genome size in humans), theoretically, a sequence longer than 16-bp is needed and this can be achieved by the consecutive linking of at least six ZF modules. Since individual zinc finger typically binds 3- or 4-bp of DNA we generated DNA-binding domains (DBDs) by assembling 6-fingers arrays directed to the selected 18-bp DNA sequences using the modular assembly method. We took advantage of an online web-

DBD N°	Target site	Gap	Strand	Linker ‡
1	gaac**ACCCCCAAT**ctcccag**ATGCTGATT**	7-bp	+	A
2	gaa**CACCCCCAA**tctccca**GATGCTGAT**t	7-bp	+	A
3	gaac**ACCCCCAAT**ctc**CCAGATGCT**gatt	3-bp	+	A
4	GAA**CACCCCCAA**tctcc**CAGATGCTG**att	5-bp	+	A
5	cttgtgggg**GTTAGAGGG**t**CTACGACTA**a	1-bp	-	B
6	cttgt**GGGGGTTAG**ag**GGTCTACGA**ctaa	2-bp	-	B
7	**CTTGTGGGGGTTAGAGGG**tctacgactaa	No Gap	-	B
8	**CTTAGGAGG**g**GGAGGTCAC**t	1-bp	+	B
9	**CTTAGGAGG**gg**GAGGTCACT**	2-bp	+	B
10	g**AATCCTCCCCCTCCAGTG**a	No Gap	-	B

‡ Linker A: see Ref. 19; Linker B see Ref. 31

Table 2. Ttarget sites in the human rhodopsin promoter recognized by the artificial ZF-TFs generated. Letters in bold represents the DNA base pairs directly recognized by the zinc fingers of the DNA Binding Domains.

resource to obtain the protein sequence of the DBD specific for a given 18-bp target site (http://www.zincfingertool.org)[176-178]. This online algorithm is extremely user-friendly. It receives as input the target DNA sequence and it gives as output the protein sequence of the DBD which putatively binds the given target sequence. Due to the lack of availability of zinc fingers for all possible triplets[173; 174; 176; 183; 184] and to enhance DNA binding affinity[185], we designed 10 ZF modules with two arms, each composed by 3 zinc finger units (arm 1 encoding fingers 1.2.3 and arm 2 encoding fingers 4.5.6, respectively) bridged together by linker peptides that enable to span up to 10-bp of DNA

gaps between their target recognition sites (**Fig. 13**). For gaps longer than 3-bp we used the structured linker reported by Moore[186], whereas for shorter gaps or continuous sequences we used the flexible linker reported by Rebar[181] (**Table 2**). We next fused the zinc finger DNA-binding domains to a nuclear localization signal (NLS), a C-Terminal HA-Tag and to effector domains that include either the herpes-simplex-virus based transcriptional activator VP64

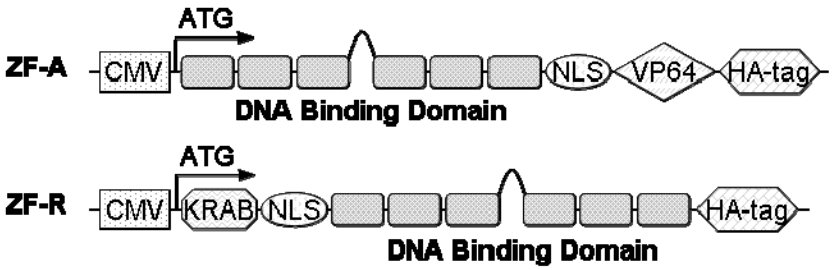

Figure 13. Schematic representation of the zinc finger transcriptional activator (ZF-A) and repressor (ZF-R)

domain[187] (Zinc-Finger transcriptional activators; ZF-A) or the human-derived Krüppel-associated box (KRAB) repression domain[179] (Zinc-Finger transcriptional repressors: ZF-R; **Fig. 13**).

1.2 *In vitro* selection of functional ZF-Rs

To select ZF-ATFs that are functional in hRHO-P transcriptional control, we first performed firefly luciferase reporter gene transactivation assays by co-transfecting HEK293 cells with both the reporter gene driven by the human rhodopsin proximal promoter (-330 to +32 relative to the transcription start site), and the individual ZF-As. Two out of the 10 constructs, significantly transactivated luciferase expression to levels comparable to those obtained with a plasmid encoding for the endogenous rhodopsin transcription activator CRX (Cone-Rod Homeobox containing gene)[188] (**Fig. 14**; n=5 from 3 independent experiments; ZF-A2, p=0.009269; ZF-A6, p=0.008928). When

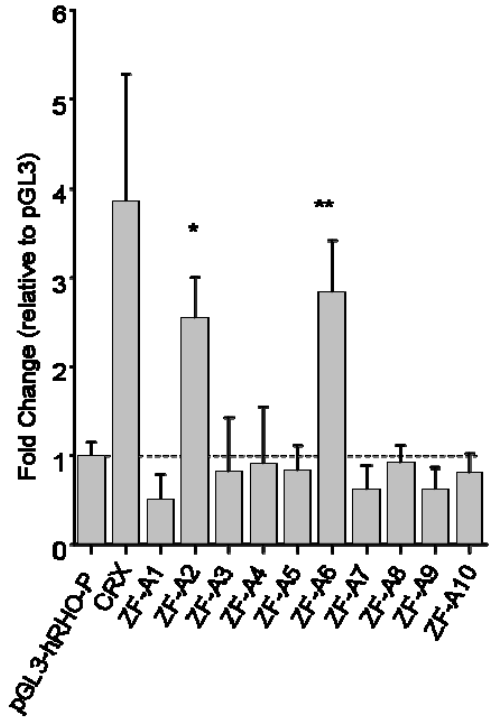

Figure 14. Histograms representing the fold change (luciferase activity relative to the control vector; pGL3-*hRHO-P*) in HEK293 cells upon transfection of the transactivator constructs (ZF-As). ZF-A2 and ZF-A6 significantly transactivate luciferase expression. As a positive control, pGL3-*hRHO-P* was co-transfected with a plasmid encoding for CRX, a known transactivator of Rhodopsin.

transfected with a luciferase expression plasmid containing the murine rhodopsin proximal promoter region (harboring several mismatches compared to the human counterpart), none of the 10 constructs were capable to transactivate reporter gene expression and results obtained for the selected functional activators ZF-A2 and ZF-A6 are shown in **Figure 15**. To evaluate ZFPs-mediated transcriptional repression, we assessed their ability to downregulate

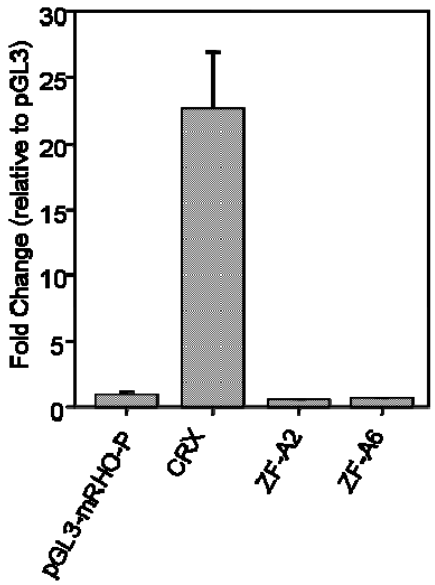

Figure 15. The functional transcriptional activator of the human rhodopsin promoter (ZF-A2 and ZF-A6) are not able toupregulate luciferase expression driven by the murine rhodopsin promoter.

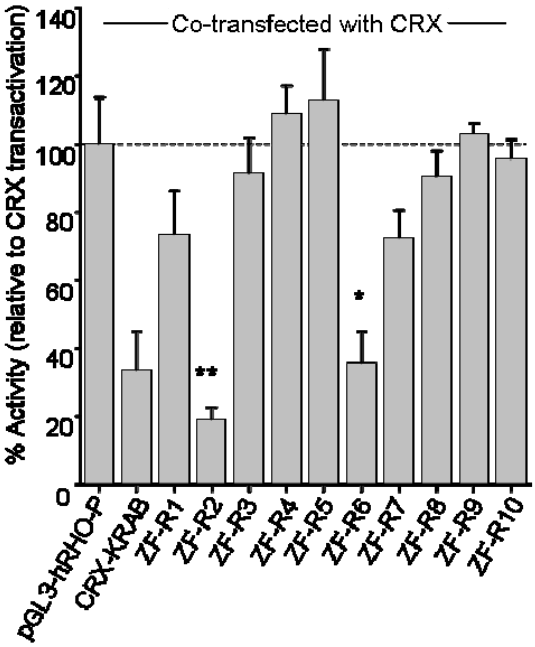

Figure 16. Histograms representing the extent of repression relative to CRX transactivation of luciferase activity mediated by ZF-Rs. ZF-R2 and ZF-R6 were able to significantly repress luciferase activity induced by CRX. As a positive control CRX DBD was fused to the KRAB repressor domain (CRX-KRAB).

CRX-mediated transcription through triple transfection in HEK293 cells including the ZF-Rs, the CRX and the reporter plasmids. Notably, ZF-R2 and ZF-R6, containing the same DBDs as ZF-A2 and ZF-A6, significantly reduced luciferase expression levels (81% and 64%, repression relative to CRX transactivation, respectively. **Fig. 16**; n=5 from 3 independent experiments; ZF-R2, p=0.000480; ZF-R6, p=0.009053) similarly to the repression obtained with CRX DBD fused to KRAB used as positive control[189]. As a further

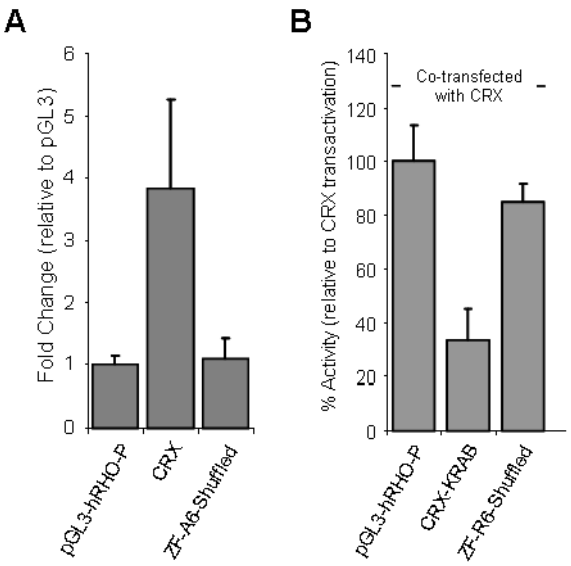

Figure 17. The artificial transcription factors with shuffled DBD derived from ZF-A6 (A) and ZF-R6 (B) are not active.

control, either activation or repression activity were completely abolished when exchanging the relative position of individual ZF units of the DBD 6 (from 1.2.3-4.5.6 to 5.1.6-3.4.2; ZF-A6- or ZF-R6-shuffled; **Fig. 17**). In addition, electromobility shift assay (EMSA) showed binding specificity of ZF-R6 (**Fig. 18**) to the target promoter sequence *in vitro*.

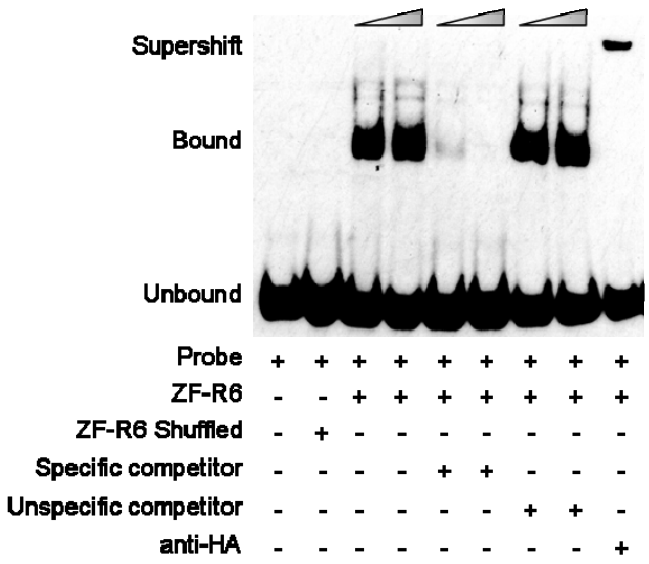

Figure 18. Binding activity of ZF-R6 assayed by electromobility shift assay (EMSA). An evident shift can be observed when an increasing amount of nuclear extract from HEK293 cells, transfected with a plasmid encoding for ZF-R6 protein, is incubated with the double stranded specific target site probe (Lane 3-4). The shift is not observed using nuclear extract from cells expressing the ZF-R6 shuffled (Lane 2). Specific binding is abolished upon addition of 10x or 100x molar excess of cold probe to the mix (Lane 5-6), whereas no effect can be detected adding 10x or 100x molar excess of an unspecific cold probe (Lane 7-8). Supershift is obtained adding an antibody recognizing the HA-tag of the ZF-R6 to the mix (Lane 9).

1.3 ZF-Rs mediated repression of human rhodopsin in retinal stem cells

To probe whether the two selected ZF-ATFs promote rhodopsin transcriptional repression in the chromosomal context, we took advantage of an *in vitro* system to differentiate retinal precursors (Retinal Stem Cells, RSC)[190]. Neurospheres derived from the murine adult ocular ciliary margin, differentiate *in vitro* into various retinal neuronal cell types, including photoreceptor precursors

expressing rhodopsin, beta-subunit of phosphodiesterase-6 (PDE6β), and cGMP-gated channel (Cnga)[191]. We explanted neurospheres from the adult P347S transgenic mouse. The aforementioned mouse model of adRP harbors a human rhodopsin transcriptional unit (including 4kb of the proximal rhodopsin promoter region) of a patient carrying a P347S mutation[94] in addition to the endogenous wild-type murine rhodopsin alleles (mRho). P347S neurospheres once differentiated into photoreceptors-like cells (Rho+ cells) underwent apoptosis with kinetics similar to that observed in another severe retinal degeneration model (rd-1 mouse[191]). Virtually all rhodopsin positive cells (Rho+ cells) were also TUNEL positive 12 days after adding the differentiating medium to P347S retinal cell precursors (**Fig. 19**). Thus, we expected that an efficient

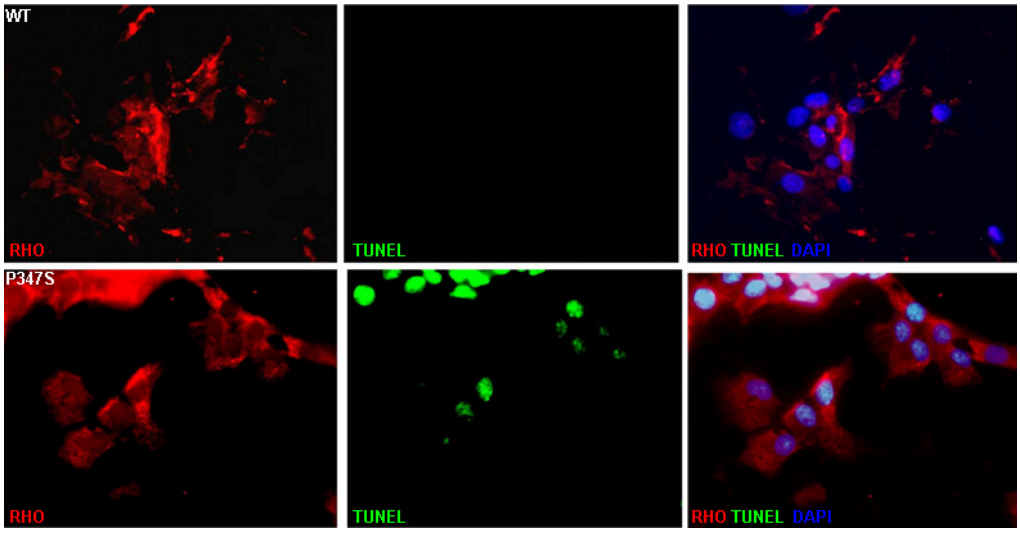

Figure 19. Apoptosis in P347S Retinal Stem Cells (RSC).Retinal stem cells from P347S and expressing rhodopsin (Rho+) spontaneously undergo apoptosis (Tunel+) 12 days after explants (Lower panels). Control RSC explanted from C57BL/6 animals are viable (Upper panels)

and specific repression of the human mutated rhodopsin, combined with the preservation of the endogenous murine rhodopsin expression, would result in rescue/survival of photoreceptor precursors. To this end, we transduced P347S retinal precursor cells 7 days after adding the differentiating medium with retroviruses encoding for the selected ZF-Rs (2 and 6) or ZF-R6 shuffled as a

negative control. Twelve days after the induction of differentiation ~70% of cells expressed rhodopsin (Rho+), 98% of which were also double positive (HA+; Rho+) confirming that retroviruses transduce retinal cell precursors efficiently[191]. ZF-R2 or ZF-R6 transduction produced a significant 77% and 92% protection from cell death, respectively, whereas virtually all the ZF-R6 shuffled transduced cells underwent apoptosis (**Fig. 20, panels A,B and D**; n=2 from 2

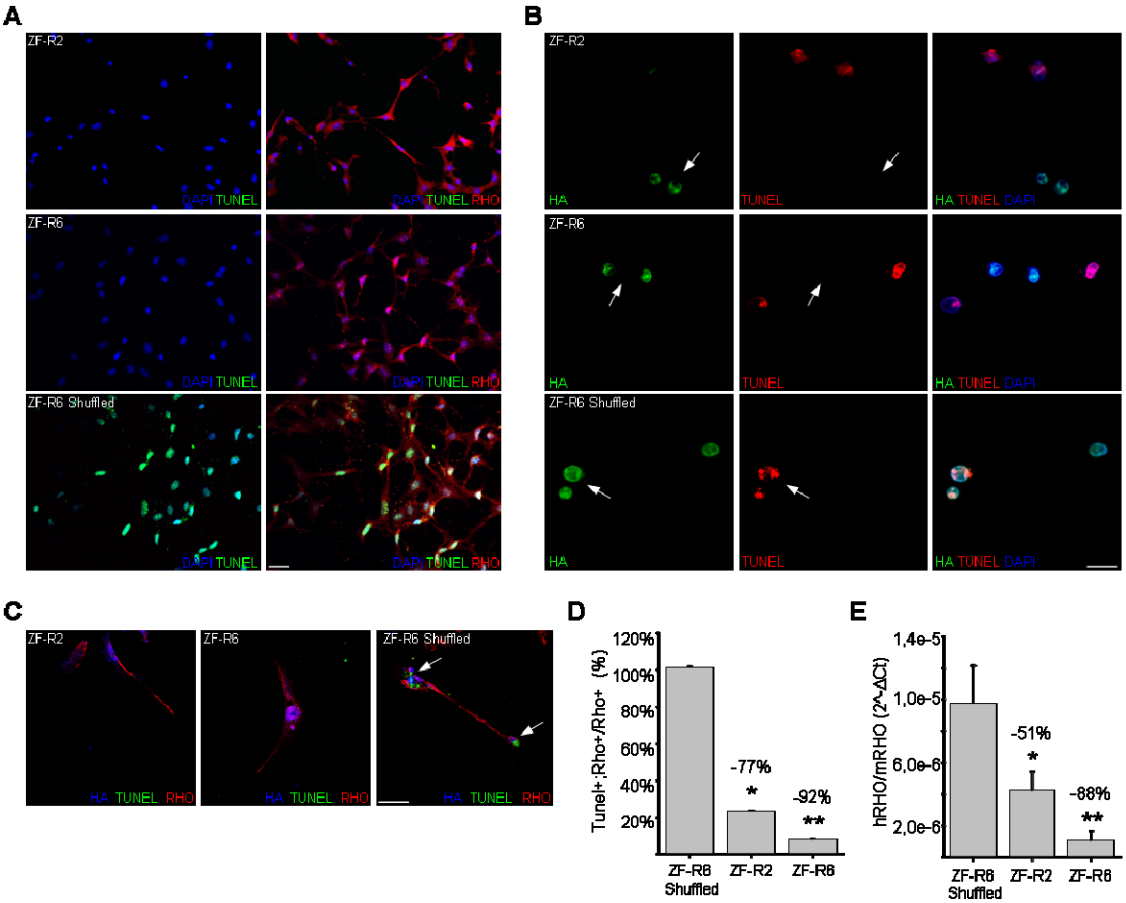

Figure 20. ZFP-mediated repression of Rhodopsin in Retinal Stem Cells (RSC). (A) Rhodopsin and TUNEL staining of RSC from the P347S mouse differentiated for 7 days, then transduced with retroviruses containing ZF-R2, ZF-R6 and ZF-R6 shuffled and analyzed after 4 days. ZF-R2 and ZF-R6 transduction prevents apoptosis (TUNEL +; green) in Rho+ cells (Red). ZF-R6 shuffled is unable to protect photoreceptor-like cells from death. (B) Immunofluorescence shows the specific protection effect of ZF-Rs. Photoreceptor-like cells are protected from apoptosis (TUNEL +; red) when transduced (HA+ cells; green) with ZF-R2 and ZF-R6 (arrows). No protection effect is observed in cells transduced with ZF-R6 shuffled. (C) Confocal images analysis shows that Rho+ (red) and HA+ (blu) cells are protected from apoptosis (TUNEL +; green) only when transduced with ZF-R2 and ZF-R6. (D) Graph showing the quantification of the result reported in Fig. 2a. (e) Quantitative RT-PCR analysis of hRHO mRNA levels in transduced cells. Values were normalized with photoreceptor-specific mRho transcript levels. (Scale bars, 100µm).

independent experiments; ZF-R2, p=4.54x10-11; ZF-R6, p=1.18x10-11). Confocal images of triple stained (HA+; Rho+; TUNEL+) samples confirmed

that only cells expressing ZF-R2 or ZF-R6 are protected from apoptosis (**Fig. 20, panel C**).

To assess whether protection from apoptosis was exerted through the specific repression of human rhodopsin transcription, we measured hRHO mRNA levels, 4 days after transduction. ZF-R2 and ZF-R6 transduced cells revealed a 51% and 88% reduction of human rhodopsin transcript levels, respectively, compared to hRHO levels in ZF-R6 shuffled treated cells (**Fig. 20, panel E**; n=2 from 2 independent experiments; ZF-R2, p=0.0133; ZF-R6, p=0.0055). These results show that ZFPs promote a strong human rhodopsin transcriptional repression in a chromosomal context, and that this, in turn, can protect retinal progenitors of an adRP model from apoptosis.

2. ASSESSMENT OF EFFICACY OF AAV-MEDIATED GENE TRASNFER OF ARTIFICIAL ZF-REPRESSOR TO PHOTORECEPTORS OF ADRP MOUSE MODEL

2.1 Delivery of ZF-Rs to murine photoreceptors decrease adRP retinal progression

The ZF-R6 construct, which provided robust human rhodopsin transcriptional repression and apoptosis protection, was selected for *in vivo* experiments. We generated an AAV2/8 vector currently considered the most efficient delivery vehicle for photoreceptor gene transfer[151; 153; 155; 182; 192], containing the ZF-R6 construct under the photoreceptor-specific Rhodopsin Kinase (RHOK) promoter element[151] (AAV2/8-RHOK-ZF-R6). In homozygous P347S mice the overexpression of the mutated rhodopsin leads to a fast progression of retinal degeneration. Indeed, by P21, only 1 row of photoreceptor nuclei was detectable by histology. Thus, in order to decrease human P347S rhodopsin expression levels we crossed P347S mice with a wild-type C57BL/6 background. The resulting progeny (P347S+/-) still overexpressed the human rhodopsin transgene ~1.4 folds compared to the expression derived by the two endogenous murine alleles (mRho) and displayed a milder retinal degeneration (**Fig. 21**).

At P70, P347S+/- retinae still retained 5-6 rows of photoreceptor nuclei and residual functionality, thus allowing the evaluation of disease progression over time by both histology and electroretinography (ERG) analysis. We first assessed transcriptional repression by subretinal co-injection in P4 P347S+/- of 1 x 10^9 genome copies (GC) of AAV2/8-RHOK-ZF-R6 and a vector encoding the Enhanced Green Fluorescent Protein (EGFP; AAV2/1-CMV-EGFP) to

identify the transduced retinal area. The contralateral eye received a mix of AAV2/1-CMV-EGFP and AAV2/8-RHOK-EGFP vectors as control. Western blot

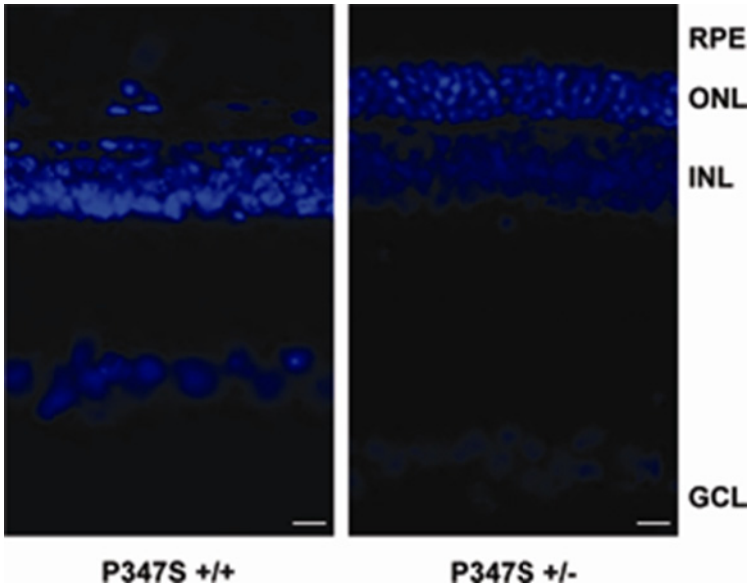

Figure 21. Morphology of P347S+/+ and P347S+/- at 1 month of age (P30).Immunofluorescence analysis shows that at one month of age only one row of photoreceptor cells nuclei (ONL) is present in the P347S+/+ retinal section (Left panel). A milder retinal degeneration can be observed in age matched P347S+/- mice (Right panel; RPE: Retinal Pigment Epithelium; ONL: Outer Nuclear Layer; INL: Inner Nuclear Layer; GCL: Ganglion Cell Layer; Scale bar, 10mm).

analysis showed expression of ZF-R6 protein in the retina twelve days post injection (P18, before photoreceptor degeneration; **Fig. 22, panel A**). Quantitative real-time PCR analysis demonstrated that the EGFP positive portion of the retina treated with AAV2/8-RHOK-ZF-R6 displayed a significant ~26% reduction of the hRHO transcript relative to the endogenous mRho compared to contralateral EGFP-treated retinae (**Fig. 22, panel B**; n=9 from 2 independent littermates; p=0.002643). In contrast, the expression profile of 3 photoreceptor specific genes was unchanged (**Fig. 22, panel B**; n=4). To evaluate whether the specific downregulation of the human rhodopsin transgene improved retinal function of the animal model, we recorded

electroretinograms (ERGs) in treated and untreated eyes one month after vector administration (P30). As shown (**Fig. 23**) the delivery of ZF-R6 to diseased photoreceptors resulted in an amelioration of visual function measured in scotopic and photopic conditions. We next evaluated the ability of AAV2/8-RHOK-ZF-R6 to impact on P347S photoreceptor disease progression.

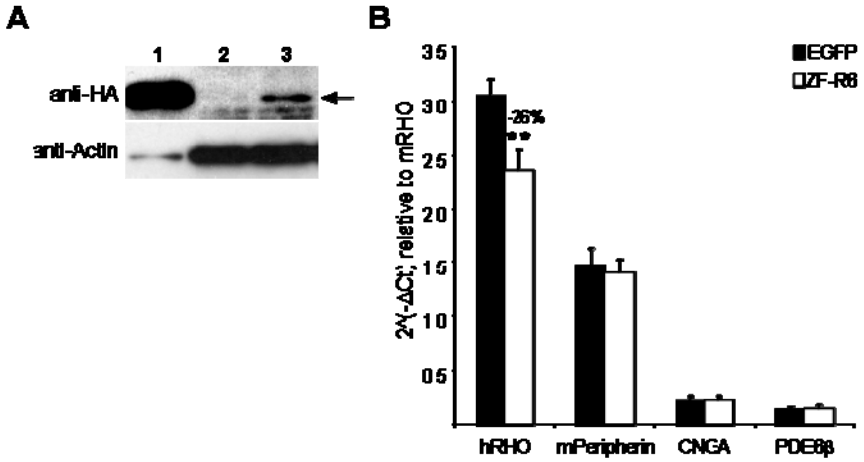

Figure 22. AAV-mediated photoreceptor ZFP gene transfer results in the downregulation of hRHO transgene. (A) Western blot analysis with anti-HA antibody of lysates from P347S retinae (P18) injected with AAV2/8-RHOK-ZF-R6 at P4. Lane 1: HEK293 cells transfected with a plasmid encoding for ZF-R6; lane 2: P347S retinal extracts transduced with AAV2/1-CMV-EGFP and AAV2/8-RHOK-EGFP; lane 3: P347S retinal extracts transduced with AAV2/8-RHOK-ZF-R6 and AAV2/1-CMV-EGFP. Actin expression was used for normalization (anti-actin antibody). (B) Quantitative RT-PCR analysis on P18 retinae transduced at P4 with AAV2/8-RHOK-ZF-R6 and AAV2/1-CMV-EGFP. A 26% specific downregulation of human rhodopsin transgene expression is measured in treated eyes (white bars) compared to control injected eyes (black bars). Photoreceptor specific gene expression levels (Peripherin, PDE6β and Cnga) remained unaltered in treated and control retinae (values are normalized using endogenous murine rhodopsin transcript levels).

Since we noticed variability in retinal function assessed by ERG among different littermates, despite similar P347S rhodopsin levels, we decided to contextually measure base-line ERG parameters and subretinally treat the animals (P30) and then monitor disease progression over time (P60 and P90). At 1 month of age in two independent littermates both scotopic and photopic ERG amplitudes were measured (a-wave max: pre-treatment EGFP=84.80 ±

9.77; pre-treatment ZF-R6=80.52 ± 10.39; b-wave max: pre-treatment EGFP=347.77 ± 30.73; pre-treatment ZF-R6=320.76 ± 23.98; n=12; **Fig. 24, panel A**). At both 30 and 60 days following subretinal vector administration, the eyes that received AAV2/8-RHOK-ZF-R6 showed a preservation of ERG responses compared to control eyes, in which the degeneration led to a ~40% reduction of the b-wave max amplitude (P60 data not shown; P90, a-wave max: post-treatment EGFP=39.60 ± 7.78, p<0.01; post-treatment ZF-R6 =55.53 ±

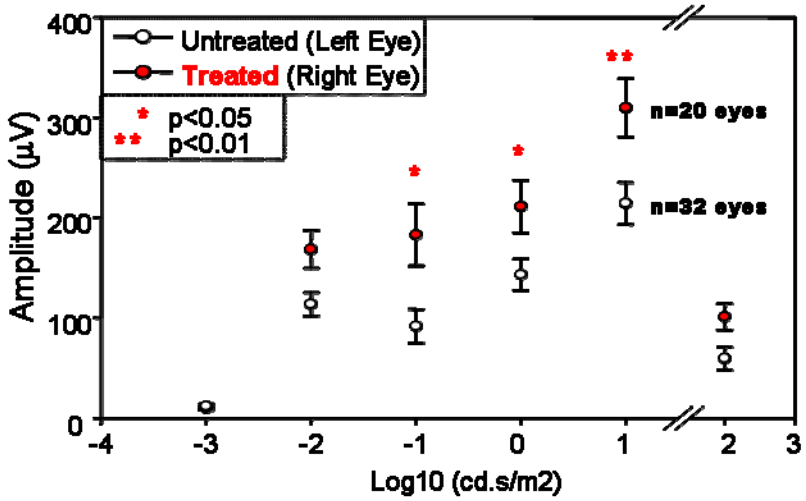

Figure 23. Functional improvement measured by electroretinogram (ERG) upon ZF-R6 delivery in diseases photoreceptors of adRP mouse model. B-wave max amplitudes under scotopic and photopic conditions of retinae treated with the therapeutic vector (AAV2/8-RHOK-ZF-R6; red circle) or with a control (AAV2/1-CMV-EGFP; white circle) measured at post natal day 30 (P30). The luminance is plotted on a log scale.

8.69; b-wave max: post-treatment EGFP=222.06 ± 24.54, p<0.01; post-treatment ZF-R6 =286.82 ± 11.90; n=12; **Fig. 24, panel A**). In addition, histological analysis of mice at P90 demonstrated that ZF-R6 expression was also associated with a partial protection of photoreceptor degeneration (**Fig. 24, panel B**; n=3). Finally, to evaluate potential toxicity on retinal function of a healthy mouse retina due to ZF-R6 treatment, we subretinally injected AAV2/8-RHOK-ZF-R6 in a cohort of 10 C57BL/6 animals. Two months after vector delivery neither histological abnormalities nor reduction both in a- and b-wave

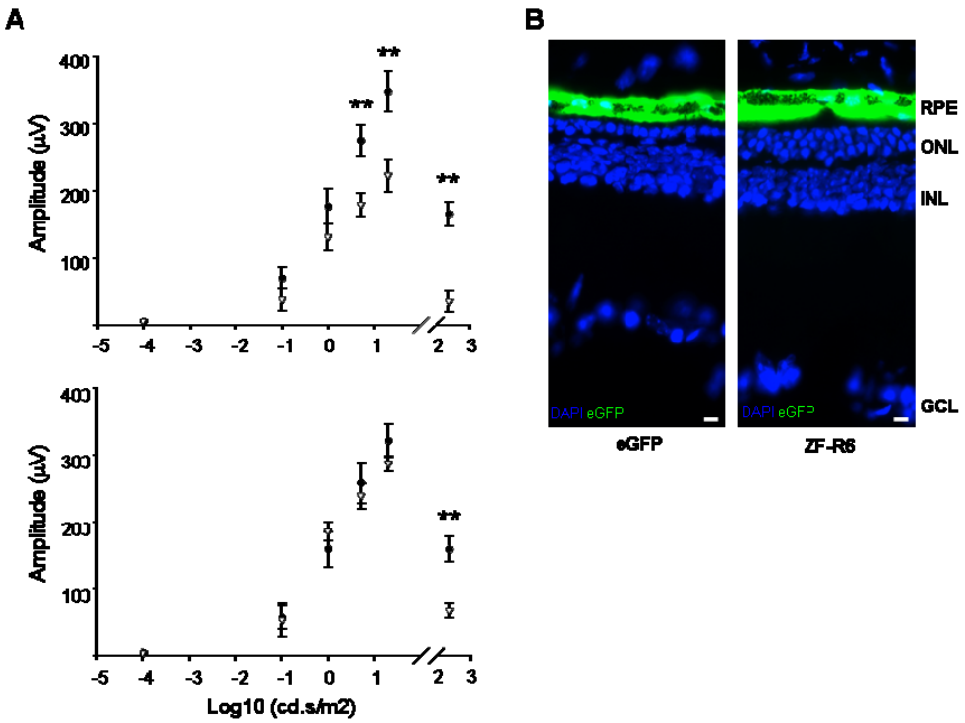

Figure 24. Delay of disease progression upon ZF-R6 delivery in diseases photoreceptors of adRP mouse model. (A) ERG analysis on P347S+/- animals subretinally injected with AAV2/8-RHOK-ZF-R6 and AAV2/1-CMV-EGFP or AAV2/1-CMV-EGFP and AAV2/8-RHOK-EGFP at P30. B-wave amplitudes under scotopic and photopic conditions before (base-line; P30; black circles, n= 12 eyes) and after treatment (P90; white circles, n=12 eyes). The kinetics of visual function loss is delayed in ZF-R6 treated retinae (lower panel) compared to control injected eyes (upper panel). (B) Immunofluorescence analysis shows that ZF-R6 transduced retinae resulted in a partial preservation of photoreceptor cell nuclei compared to EGFP injected eyes. (RPE: Retinal Pigment Epithelium; ONL: Outer Nuclear Layer; INL: Inner Nuclear Layer; GCL: Ganglion Cell Layer; Scale bar, 10µm).

could be measured in treated eyes compared to untreated parental eyes supporting the safety of this approach (**Fig. 25**).

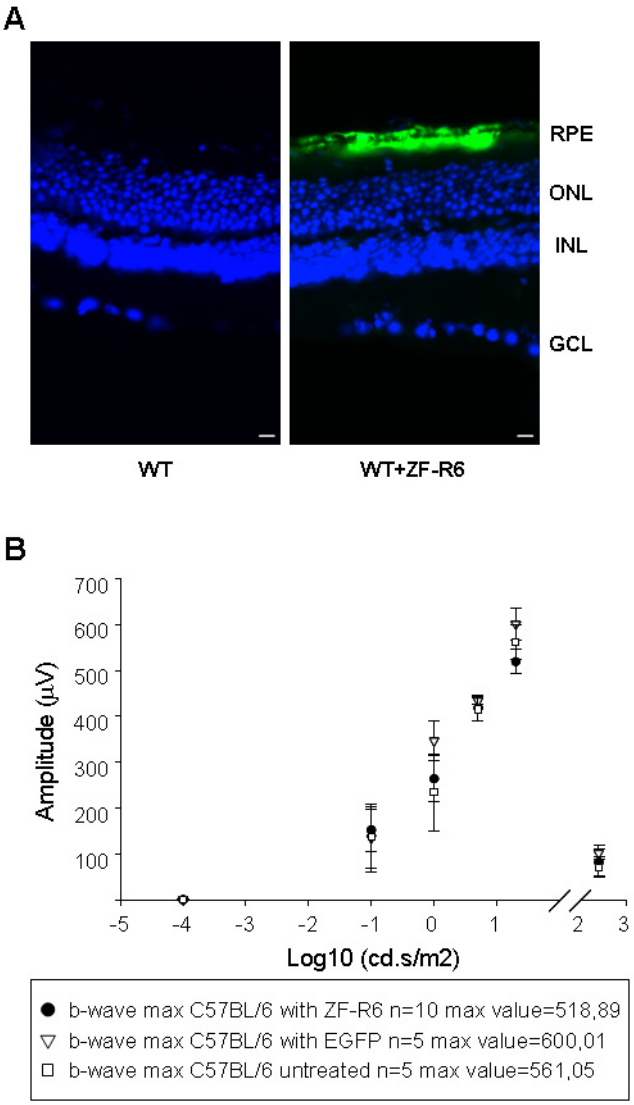

Figure 25. C57BL/6 mice treated with ZF-R6. (A) Immunofluorescence analysis shows that no detrimental effect can be observed in retinal morphology one month after AAV-mediated delivery of ZF-R6 (AAV2/8-RHOK-ZF-R6) to retinal photoreceptors. (B) Electroretinogram (ERG) analysis shows that no abnormality can be measured in the b-wave amplitude of C57BL/6 mice retinae two months after photoreceptor delivery of the ZF-R6 (Black filled circles) compared to EGFP treated (White triangles) or untreated (White squares) retinae. (RPE: Retinal Pigment Epithelium; ONL: Outer Nuclear Layer; INL: Inner Nuclear Layer; GCL: Ganglion Cell Layer; Scale bar, 10µm)

Discussion

DISCUSSION

Gene therapy for the treatment of recessive inherited ocular disorders has proven successful. In 2008, the results published for three independent clinical trials carried out to treat Leber's congenital amaurosis (LCA) due to *RPE65* mutations provided sound evidence for the use of gene therapy approaches to treat recessive ocular diseases[62-68]. By contrast, the development of therapeutic strategies to treat autosomal dominant retinal disorders has not proceeded at the same pace, largely due to their complexity. The common approaches under consideration for the therapy of these genetic disorders include strategies to modulate secondary effects associated with disease, such as factors influencing cell longevity or function[193-195]. However, many dominantly inherited disorders may require correction of the underlying genetic defect and the common strategies to this purpose mainly rely on the silencing of the disease gene expression through RNAi-, ribozyme- or miRNA-based approaches. An alternative strategy is based on the transcriptional repression of a disease gene using artificial transcription factors which were developed in the last ten years with the aim to create molecules tailored to control the expression profiles of a disease-causative gene.

In this study we demonstrated that photoreceptor delivery of an AAV2/8 vector containing an artificial zinc finger-based transcriptional repressor (ZF-R6) targeted to the human rhodopsin promoter robustly repressed the target gene expression, and this in turn was associated with improvement of photoreceptors functionality and in a delay of the disease progression in P347S mouse model of autosomal dominant *Retinitis Pigmentosa* (adRP).

The first part of this PhD project was aimed at generating functional artificial transcription factors (ATFs) targeted to human rhodopsin promoter. To this end, we first identified the DNA sequence in the proximal rhodopsin promoter to serve as target for the ATFs. We established two criteria to select the target sequences: i) the absence of polymorphisms and ii) the accessibility of the target site in the chromatin environment that can both affect the binding of the ATFs. Respecting these criteria, we identified ten putative target sites in the human rhodopsin proximal promoter in a region where no polymorphisms are annotated and in proximity of binding sites for known transcriptional regulators of the rhodopsin gene. In addition, for experimental needs, we chose target sites in a region which is not conserved between rodents and humans genomes. The reason of this choice derives by the animal model available for our *in vivo* studies, the P347S mouse model. This animal carries a P347S human mutated rhodopsin allele in addition to the murine endogenous alleles. Targeting a sequence which is not conserved between mouse and man will allow us to be selective in modulate human rhodopsin expression leaving unaltered the endogenous counterpart.

We identified ten putative target sites in the proximal rhodopsin promoter matching the criteria explained above and, using the modular assembly method, we generated ten zinc-finger based transcriptional modulator of the rhodopsin gene. We assessed *in vitro* the functionality of these ATFs by reporter assays and we identified two out of ten activators and repressors able to control rhodopsin promoter activity. Since our final goal is to downregulate the expression of mutated human rhodopsin causing adRP, the functionality of the selected repressors (ZF-R2 and ZF-R6) was further validated in another *in vitro* system which better mimic the real condition: retinal stem cells (RSCs)

explanted by the P347S mouse model. Indeed, the reporter system used in the first screening had the main limitation to be based on an episomal system transfected in the cells which is largely far from the condition in which the promoter to be controlled is in its chromatin environment. On the other hand, RSCs explanted by the P347S mouse model have the advantage to harbor the human rhodopsin cassette in the chromosomal context thus allowing a more accurate validation of the ATFs functionality as compared to the episomal system used in the first round of selection. Furthermore, the binding ability to the intended target site was demonstrated by Electromobility Shift Assay (EMSA).

The following part of my PhD project was to evaluate the effect *in vivo* of the delivery of the selected ZF-R6 to photoreceptors of both a mouse model of adRP (P347S) and of a normal mouse model (C57BL/6) in order to address both the efficacy and potential safety concerns, respectively. To this end, ZF-R6 was enclosed in an AAV2/8 vector, considered to date the most efficient in murine photoreceptor transduction[151] and delivered subretinally in diseased or healthy retinae. We assessed no detrimental effects, both on the functionality (measured by electroretinograms, ERG) and in the structure (evaluated histologically) of normal retinae after the treatment, suggesting that the approach is safe. Interestingly, the diseased retinae showed a preservation of retinal function compared to controls upon injection of P347S animals at birth. This result was also confirmed in adult animals in which we monitored the disease progression over time upon ZF-R6 delivery at one month of age. Indeed up to three months after the treatment, in the control-treated eyes we recorded a 40% reduction of visual function compared to the baseline while in the study-group eyes, the drop was only 10%.

As a consequence, our findings extend the possible uses of artificial zinc finger-based DNA binding proteins as a new paradigm in gene therapy to treat dominant diseases based on the modulation of an endogenous gene expression.

The common therapeutic approach to date for the treatment of gain-of-function mutations mainly rely on RNAi-mediated downregulation of a gene-causing a disease. The first example of such therapeutic strategy *in vivo* was assessed to silence a dominant allele of ataxin-1 in order to treat a spinocerebellar ataxia type 1 (SCA1) in mutational independent manner and then applied to other mouse models of human disease including the P347S adRP mouse[73; 74]. Thus, based on the data presented here, either transcriptional (artificial ZFP technology) or post-transcriptional (RNAi) silencing strategies may be considered alternatives to one another; however, a side-by-side comparison is needed to determine which of the two systems is the safest and the most efficacious. In principle, ZFP technology has the advantage that the target sequences are present in one or two copies/diploid genome, whereas the mRNA transcripts (the RNAi targets) are highly abundant. It is worth emphasizing that rhodopsin is expressed extremely highly in the retina with 6.4 million rod photoreceptor cells, each producing a steady-state level of 60 million of rhodopsin molecules (550–650 pmol)[196]. Moreover, besides the off-target silencing events which potentially set hurdles for the development of a safe ZFP- or RNAi-based system, studies have shown that high levels of siRNA may cause cellular toxicity through various mechanisms. Fatality in mice was associated with the downregulation of liver-derived microRNAs (miRNAs), indicating possible competition of the latter with shRNAs for limiting cellular factors required for the processing of various small RNAs[197; 198]. Also, an

attractive and therapeutic valuable feature of ZFPs is the possibility to control their expression levels through the use of inducible promoter systems which have not yet been optimized to control RNAi-based repression systems. Indeed, a key safety and efficacy element for the success of silencing strategies relies on fine-tuning control of the expression levels of the repressor system. In addition, a considerable potential advantage of ZF-mediated transcriptional control of an endogenous gene includes the ability to regulate all the natural splice variants of the target with a single therapeutic molecule. This of course is not the case of rhodopsin but it has already been demonstrated to be an important advantage in other therapeutic approaches[181]. Finally, the versatility of ZFPs design theoretically allows to switch the repressor with an activator domain (maintaining the same DBD) to treat also dominant diseases due to haploinsufficiency.

Mutation-independent approaches such as the strategy exploited in this study for rhodopsin linked adRP, are extremely valuable technically and economically as a single therapeutic agent that may provide a remedy for many different mutant alleles of a disease gene (for example in the case of rhodopsin, more than 150 different mutations have been identified in its sequence).

However, although this strategy may provide a rational solution for many dominant conditions, the application of the approach is complex. Indeed, one limitation of the mutational-independent silencing is the simultaneous repression of both the mutated and the normal alleles. As a consequence, a therapeutic strategy based on such approach must consider the development of a two-components system able to suppress both mutant and wild-type alleles of a target gene and simultaneously to provide a replacement gene that encodes wild-type protein but which is refractory to suppression.

The results presented here represent an *in vivo* demonstration that suppression through an artificial transcription factor and replacement given by the endogenous murine gene may provide benefit in an animal model of a dominant retinal disease. Thus, one future development will be to determine whether, in an amenable mouse model (ideally, harboring both a mutated and a normal allele of the human rhodopsin gene on a murine rhodopsin knock-out background), down-regulation of both alleles will be sufficient to inhibit/delay the disease progression or whether simultaneous replacement with a human rhodopsin transgene not sensitive to the treatment would be necessary. In the latter case, a vector incorporating both the artificial ZF-based repressor and the replacement gene ensuring their simultaneous expression will be required (for instance with bidirectional promoters enabling the coordinate expression of the two transgenes[199]). Moreover, it has been shown that either high or low rhodopsin expression levels can be deleterious to rod function, suggesting that tight regulation of rhodopsin expression levels is required for successful application of retinal repression-replacement strategy.

Our data demonstrate that the novel and generally applicable mutational-independent strategy based on artificial zinc finger proteins enables the transcriptional control of an endogenous causative gene and has perspectively therapeutic relevance for the treatment of diseases due to gain of function mutations.

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