



Transient CRISPR-Cas delivery for therapeutic gene editing in the retina

Catherine Botto

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Sorbonne Université

Ecole doctorale Cerveau Cognition Comportement (ED158)

Institut de la Vision

Transient CRISPR-Cas delivery for therapeutic gene editing in the retina

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Thèse de doctorat en Neurosciences

Dirigée par **Deniz DALKARA**

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A mes grands-parents, à mes parents,

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- American Society of Gene and Cell therapy annual congress (ASGCT) 2022 (poster, Annex 2)
- CRISPR and Translational medicine congress 2022 (oral presentation)
- International Usher Info Symposium 2021 (oral presentation)
- 10 years of Vision Institut congress 2019 (oral presentation)

I diffused my work to larger public with:

My participation to “Ma these en 180s” - Finale Sorbonne Université.

An Art animation that explain my thesis project done with Cécile Botto, my big sister, under the initiation of the Vision Institute (follow the link: <https://youtu.be/g0NeDjXlNkM>)

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List of abbreviations

AAV	Adeno-Associated virus
ABCA4	ATP-binding cassette subfamily A member 4
AD	Autosomal dominant
AMD	Age macular degeneration
ASOs	Antisense oligonucleotides
BER	Base Excision Repair
CB	Cell body
CBE	Cytosine base editors
CC	Connecting cilium
cGMP	Cyclic guanosine monophosphate
crARN	CRISPR ARN
CRISPR Cas	Clustered Regularly Interspaced Short Palindromic Repeats associated protein Cas
CRBP	Cellular Retinol Binding Protein
dCas9	Dead Cas9
DSB	Double-strand DNA breaks
ECM	Extracellular matrix
EMA	European Medicines Agency
ERG	Electroretinogram
FDA	Food and Drug Administration
GCL	Ganglion cell layer
GFP	Green fluorescent protein
GRK	G-protein coupled receptor kinase
HDR	Homology-directed repair
HITI	Homology-independent targeted integration
INL	Inner nuclear layer
ILM	Inner limiting membrane
IPL	Inner plexiform layer
IPM	Interphotoreceptor matrix
IRBP	Interstitial Retinal Binding Protein
IRD	Inherited retinal disease
iPSC	Induced pluripotent stem cell
IS	Inner segment
LCA	Leber congenital amaurosis
MLV	Murine leukemia virus

MMEJ	Microhomology-mediated end-joining
NC	Nanocapsules
NER	Nucleotide Excision Repair
NFL	Nerve fiber layer
NGS	Next Generation sequencing
NHEJ	Non-homologous end joining repair
NP	Nanoparticles
OCT	Optical coherence tomography
OLM	Outer limiting membrane
ONL	Outer nuclear layer
OPL	Outer Plexiform layer
OS	Outer-segment
PAM	Protospacer adjacent motif
PBS	Phosphate-buffered saline
PDE	Phosphodiesterase
PE	Prime editor
PL	Photoreceptor layer
PEG	Polyethylene glycol
pegRNA	Prime editing guide RNA
PR	Photoreceptors
RCD	Rod-cone dystrophies
rd10	Retinal degeneration 10 (mouse model)
RdCVF	Rod-derived Cone Viability Factor
RDH12	Retinol dehydrogenase 12
RGC	Retinal ganglion cell
RHO	Rhodopsin
RNP	Ribonucleoprotein
RP	Retinitis pigmentosa
RPE	Retinal epithelium pigment
RPE65	Retinal pigment epithelium-specific 65kDa
SaCas9	Staphylococcus aureus Cas9
SgRNA	Single guide RNA
SLN	Solid lipid nanoparticles
SpCas9	Staphylococcus pyogenes Cas9
TracrARN	Trans activating CRISPR ARN
USH	Usher syndrome
WT	Wild-type

Introduction

I. Visual system and the retina

1. Anatomy of the visual system

1.1 Visual system

The visual system is composed of a sensory organ, the eye, connected by the optic nerve to the brain. The eyes have the role to detect and adjust light stimuli in order to convert it to electric signals that will be process by the visual cortex of the brain.

Light passes through the cornea, the crystalline lens and the pupil then the vitreous before reaching the light sensor of the visual system, the retina, at the back of the eye. The retina is composed of the neural retina and the retinal pigment epithelium (RPE), a layer of epithelial cells. Capillaries originating from the central retinal artery nourish the inner part of the retina. The outer part of the retina receives its blood supply from the choroid, a highly vascularized conjunctive tissue that is just against the RPE.

Eyes present some anatomical differences between species. Rodents, non-human primate and human eye differ in their sizes and thus their volume (**Figure 1**). Most importantly, their retinas present differences that will be display in the following paragraphs focus on the retina structure and cell population.

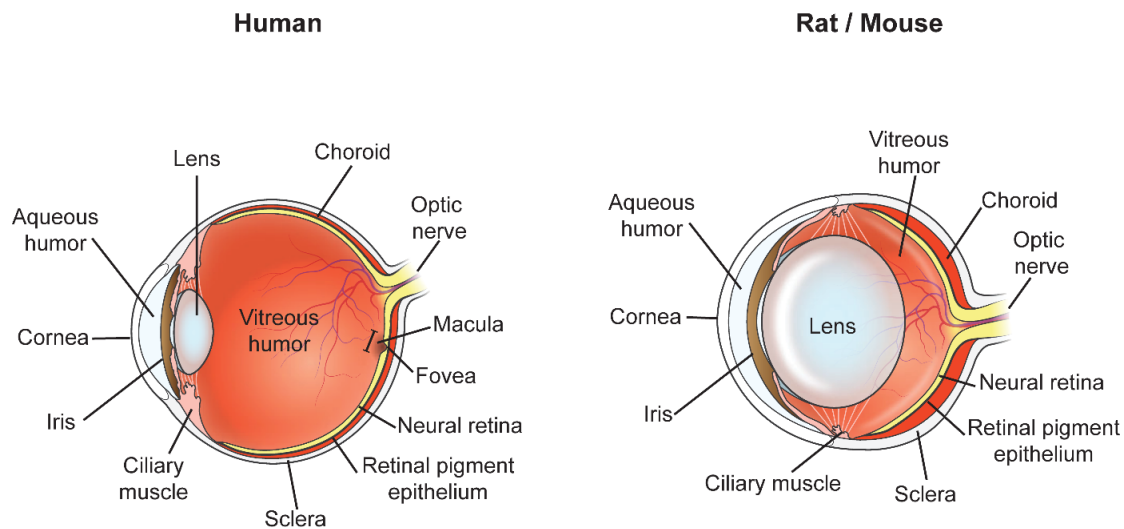


Figure 1 : Anatomical structure of human and rodent eye.

Adapted from (Veleri et al. 2015).

1.2 Structure of the retina

The vertebrate neuro-retina, a 0.5 mm-thick tissue lining the back of the eye, consists of three layers of nerve cell bodies and two layers of synapses (Malhotra et al. 2021; Scholl et al. 2016). The outer nuclear layer contains the cell bodies of the photoreceptors (PRs); the inner nuclear layer contains the cell bodies of the bipolar, horizontal and amacrine cells, and the ganglion cell layer contains the cell bodies of the retinal ganglion cells (RGC) and displaced amacrine cells. The retina is separated from the vitreous by the inner limiting membrane (ILM). On the opposite end, photoreceptors cell bodies are delimited by an outer limiting membrane (OLM) with cone cell bodies located right below it (**Figure 2**).

The two types of photoreceptors, named rods and cones, are the primary neurons of the retina. As mentioned, these light-sensing neurons lie outermost in the retina, against the RPE. Light must, therefore, travel through the thickness of the retina to reach and activate the rods and cones. Rods are spread across retinal regions and are responsible for night and peripheral vision. On the contrary, cone cells display a greater concentration in the fovea (or “area centralis” in non-primates), a unique structure that allows high-acuity vision. Primates are the only mammalian species who have a fovea.

Visual pigments are present in the outer segment (OS) of the photoreceptors. They absorb photons and thus trigger the phototransduction biochemical cascade, creating changes in electrical potentials of photoreceptor cells that are conveyed across all the inner retinal neurons of the retina. Neural processing by these layers culminates in the inner plexiform layer, where the message concerning the visual image is transmitted to the brain as spike trains from the ganglion cells through the optic nerve. Importantly, horizontal cells and amacrine cells control and modulate responses of, respectively, bipolar and ganglion cells.

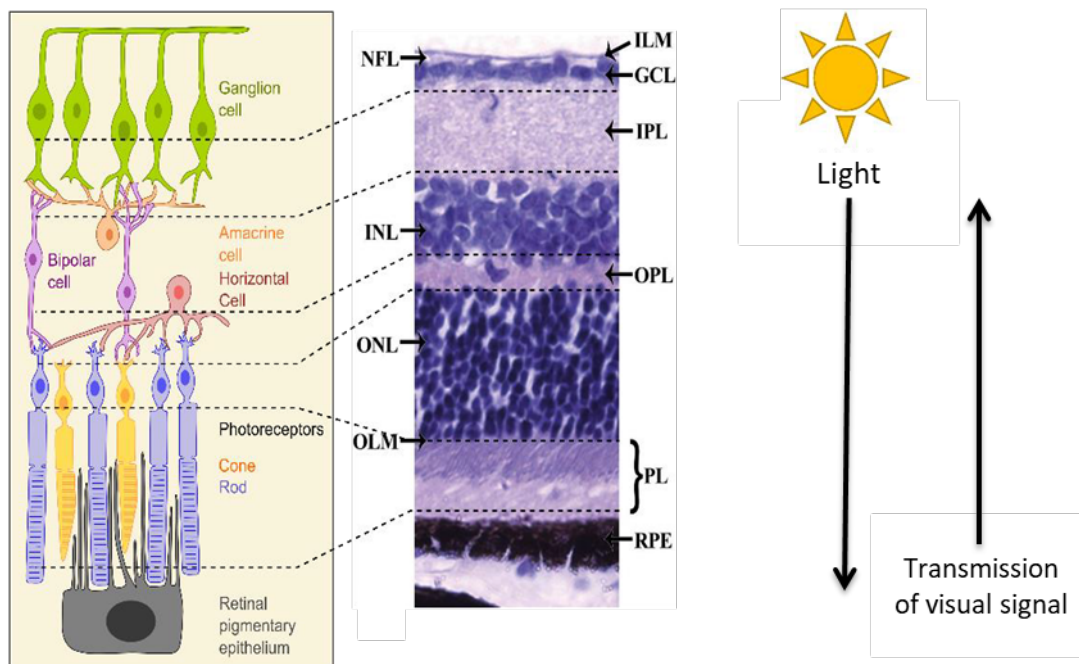


Figure 2 : The structure of the eye and the retina.

Schematic representation of retina and cell type diversity and Cross-section of human retina extracted from (Nathans et al. 1986). (Inner limiting membrane (ILM); nerve fiber layer (NFL); ganglion cell layer (GCL); inner plexiform layer (IPL); inner nuclear layer (INL); outer plexiform layer (OPL); outer nuclear layer (ONL); outer limiting membrane (OLM); photoreceptor layer (PL), and retinal pigmented epithelium (RPE) monolayer. Light path is at the opposite direction of visual message. (Adapted from (Verschueren 2019).

2. Photoreceptors

2.1 Structure of the photoreceptors

Photoreceptors can be divided in five structural and functional regions:

1. An outer segment (OS) composed of tightly packed membrane discs containing visual pigments and proteins involved in the conversion of photons to changes in membrane potential, process defined as the phototransduction cascade.
2. A connecting cilium (CC), a bridge providing the only route between the OS to the IS.
3. An inner segment (IS) containing cellular organelles as mitochondria and ribosomes.
4. A cell body (CB) with the nucleus.
5. A synaptic terminal responsible for glutamate-mediated signaling to the inner cells with a high exocytosis and endocytosis activities (LoGiudice and Matthews 2007).

To avoid light-dependent metabolic stress, and to constantly ensure maximum photosensitivity, photoreceptor cells renew their outer segments daily. Approximately 10% of rod outer segment tips are shed and digested by the RPE each day. Newly formed discs are continuously incorporated at the base of OS. (Malhotra et al. 2021).

Photoreceptors outer segments differ between cones and rods in their OS architecture, their photopigment molecules and thus in their function (**Figure 3**). Rods are involved in vision during dim-light conditions, and are extremely sensitive to light with a detection threshold of a single photon (Ingram et al. 2016). The structure of the rod outer segments provides a large surface area to increase the number of opsins involved in light absorption, which is especially important in low-light conditions. Rhodopsin, the rod visual pigment, absorbs maximally at wavelength of 500 nm.

Cones have smaller outer segments with disks formed by invaginations of the plasma membrane, unlike the fully enclosed rod disks. They are primarily involved in vision in bright light conditions. Cones have faster kinetics during phototransduction allowing perception of finer details and more rapid changes in images than in rod based vision. Cones are also the cell type responsible for color vision based on different cone opsin photopigments. The different opsins found in different cone types are sensitive to different wavelengths of light.

In humans, there are three cone subtypes: S, which are sensitive to short “blue” wavelengths (440 nm); M, which are sensitive to middle “green” wavelengths (535 nm); and L, which are sensitive to long “red” wavelengths (565 nm) (Schnapf et al. 1988).

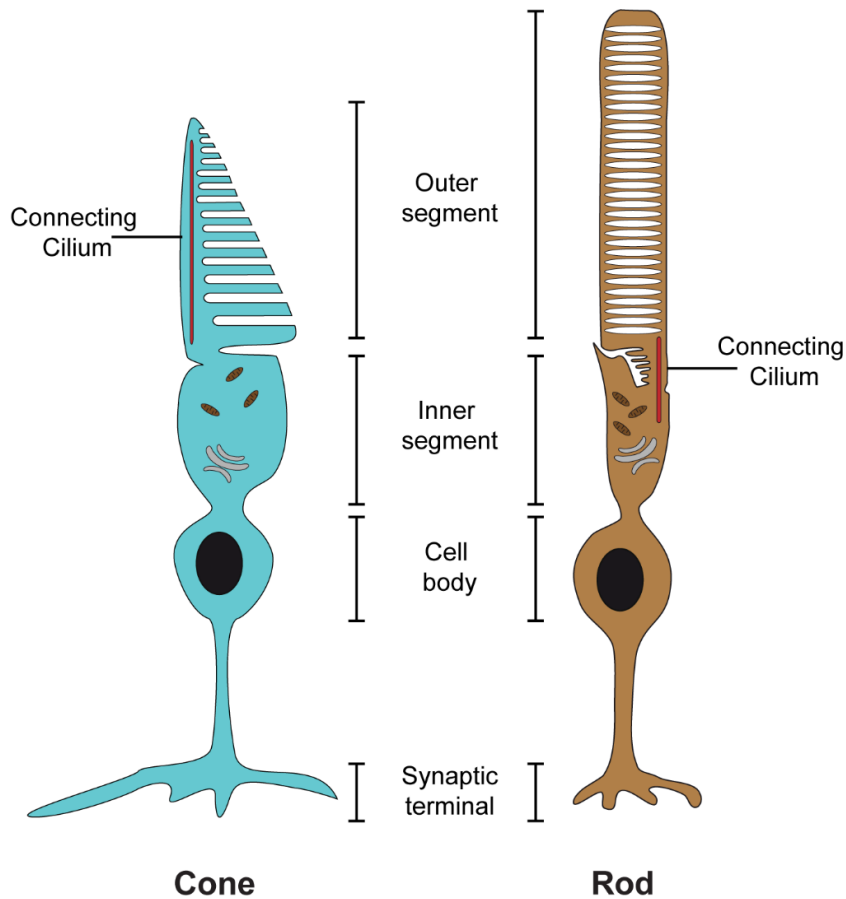


Figure 3 : Schematic representation of the two types of photoreceptors: cones and rods.

Rods are characterized by longer outer segments but smaller synaptic terminals compared to cones. Note that cones have a robust conical shape at the level of their inner segment, in contrary to rods which are thin in this area (Gagliardi 2018).

Interspecies differences in photoreceptors

Photoreceptors of primates and rodents display some specificity in terms of their size, distribution and their pigment absorption spectrum. These differences reflect functional needs and evolutionary adaptation to a specific environment.

Among mammals, cone density of primates is much higher in the central retina while rod density is higher in the periphery. This cone-enriched area named macula is not found in rodent. The high acuity vision found in primates is due to the presence of a unique region inside the macula, denominated fovea. This avascular region oxygenated by the choroidal circulation contains only cones with a peak in its center defined as the foveola (**Figure 4**).

Despite this region represent only 1% of the total retinal surface, 75% of the light entering the eye converges on this region. Moreover, RGCs and inner retinal cells in the foveola allow light to reach directly photoreceptors without being scattered. The fovea circuitry provides an optimized solution for high visual acuity with one single cone connected to a single bipolar cell, connected to a single ganglion cell.

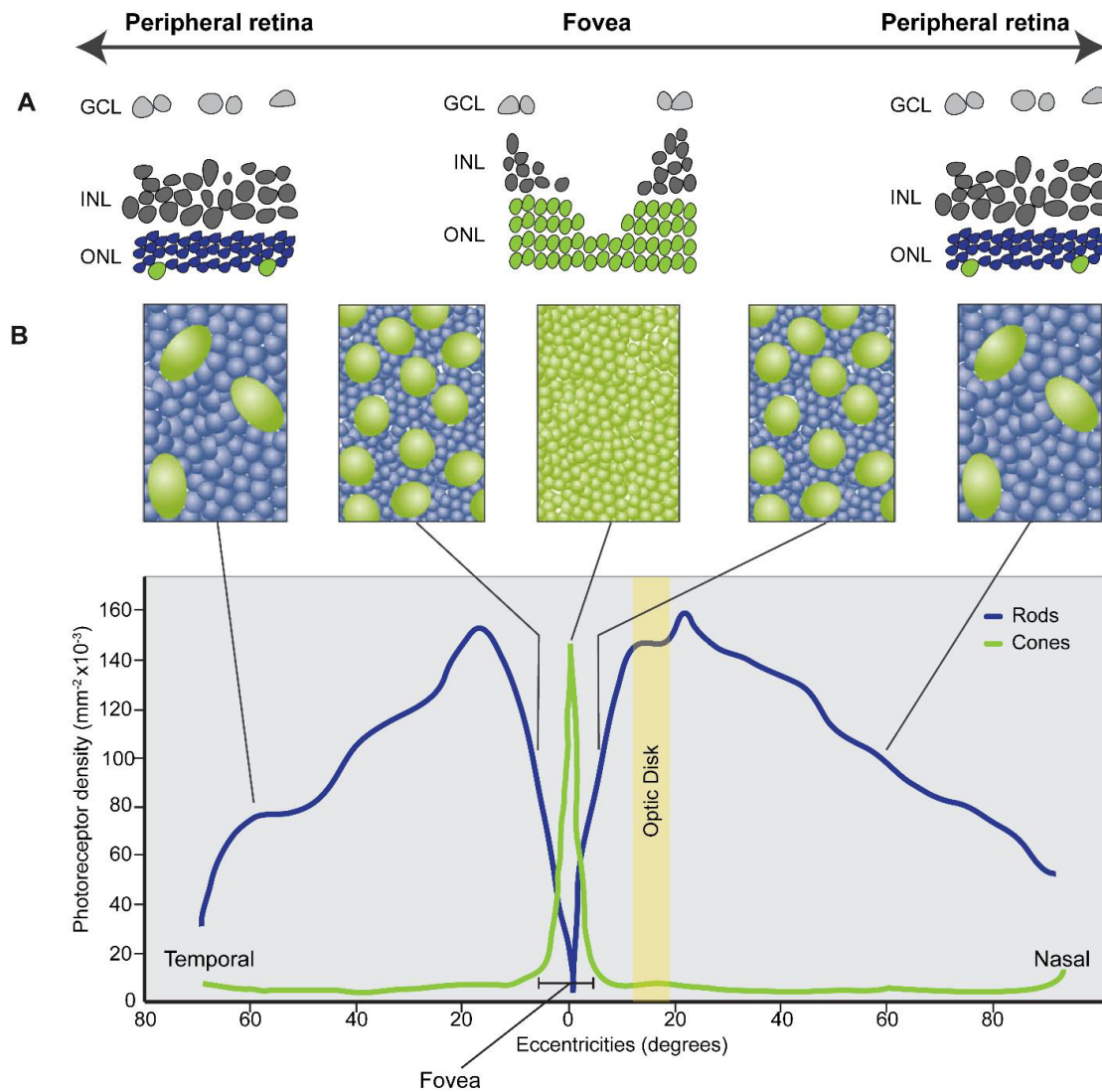


Figure 4 : The fovea, a unique structure only found in primate.

A/Schematic representation of cross-sections of peripheral and central retina. Fovea contains only cones without any other retinal cell types. B/ Distribution of rod and cone along the naso-temporal retinal axis. Adapted from (Mustafi et al. 2009)

In addition to the foveal region, an anatomical structure named calyceal processes is found in primates (**Figure 5**). Its role in the retina is not well known, however mutations in proteins located in this region leads to retinal diseases such as Usher syndrome (developed in part II).

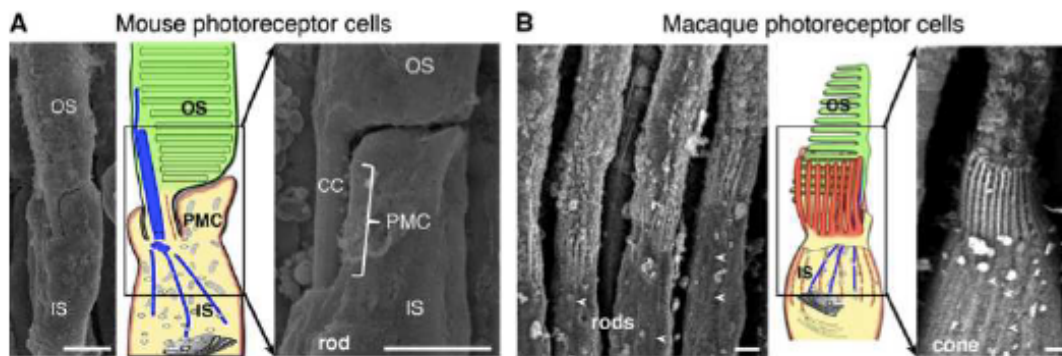


Figure 5 : Calyceal processes found only in the primate retina.

A/ Absence of calyceal processes in mouse photoreceptors. B/ Calyceal process present in macaque photoreceptors at the junction between inner and outer segments. OS: outer segment, IS: inner segment, CC: connecting cilia, PMC: periciliary membrane complex. From (Iman Sahly et al. 2012b).

In Rodents, rods that represent 97% of total photoreceptors display an inverted nuclear architecture with heterochromatin condensed at the center of the nucleus and euchromatin at its periphery. This unique pattern maximizes light detection by reducing the scattering of light in the ONL and thus the loss of photons (Solovei et al. 2009).

2.2 Functional properties of photoreceptors and retinal circuitry

Phototransduction cascade occurs within membrane discs of the rods' and cones' apical regions. In absence of light, there is a constant influx, known as dark current, of sodium and calcium ions across the cGMP-gated channels (CNG α 1/ β 1). In this dark-depolarization status, a continuous release of the glutamate neurotransmitter is maintained at photoreceptor synaptic terminals.

Incoming light directly activates the light-sensitive opsins, rhodopsin (in rods) and cone opsin. Indeed, rhodopsin and cone opsins are coupled to a chromophore derived from the vitamin A, the 11-cis-retinal. When hit by a photon, the chromophore is converted into all-trans-retinal causing a conformational change in the opsin, ultimately leading to cellular hyperpolarization. The catalytically activated opsin or rhodopsin protein binds to transducin, a G protein. Activation of transducin results in the dissociation of its α subunit, which in turns can activate the membrane-associated phosphodiesterase (PDE). cGMP hydrolysis into

GMP by PDE leads to intracellular cGMP decrease and the closure of the cGMP-gated (CNG) channels. Hyperpolarization occurs and leads to graded modulation of glutamate release at the photoreceptor synaptic terminals.

Initial status needs to be re-established to ensure photoreceptors' answer to another light stimulus. Several mechanisms occur in the photoreceptor as the self-inactivation of the transducin and the phosphorylation by the rhodopsin kinase of the opsin leading to its inactivation by the arrestin protein. However, regeneration of all-trans-retinal to its cis isomer cannot occur in the photoreceptors due to the lack of cis-trans isomerases and will need multiple steps. First, all-trans-retinal is transformed in the photoreceptors cytoplasm in all-trans-retinol by a retinol dehydrogenase (RDH12). Then all-trans-retinol is transported to the RPE by Interstitial Retinal Binding Protein (IRBP) to be recycled in 11-cis-retinal and then transported back by the same transporter to the disc membrane of the photoreceptors **(Figure 6)**. For cones visual cycle, it is müller cells that recycle all-trans-retinol visual chromophore back to 11-cis-retinol (Morshedien et al. 2019).

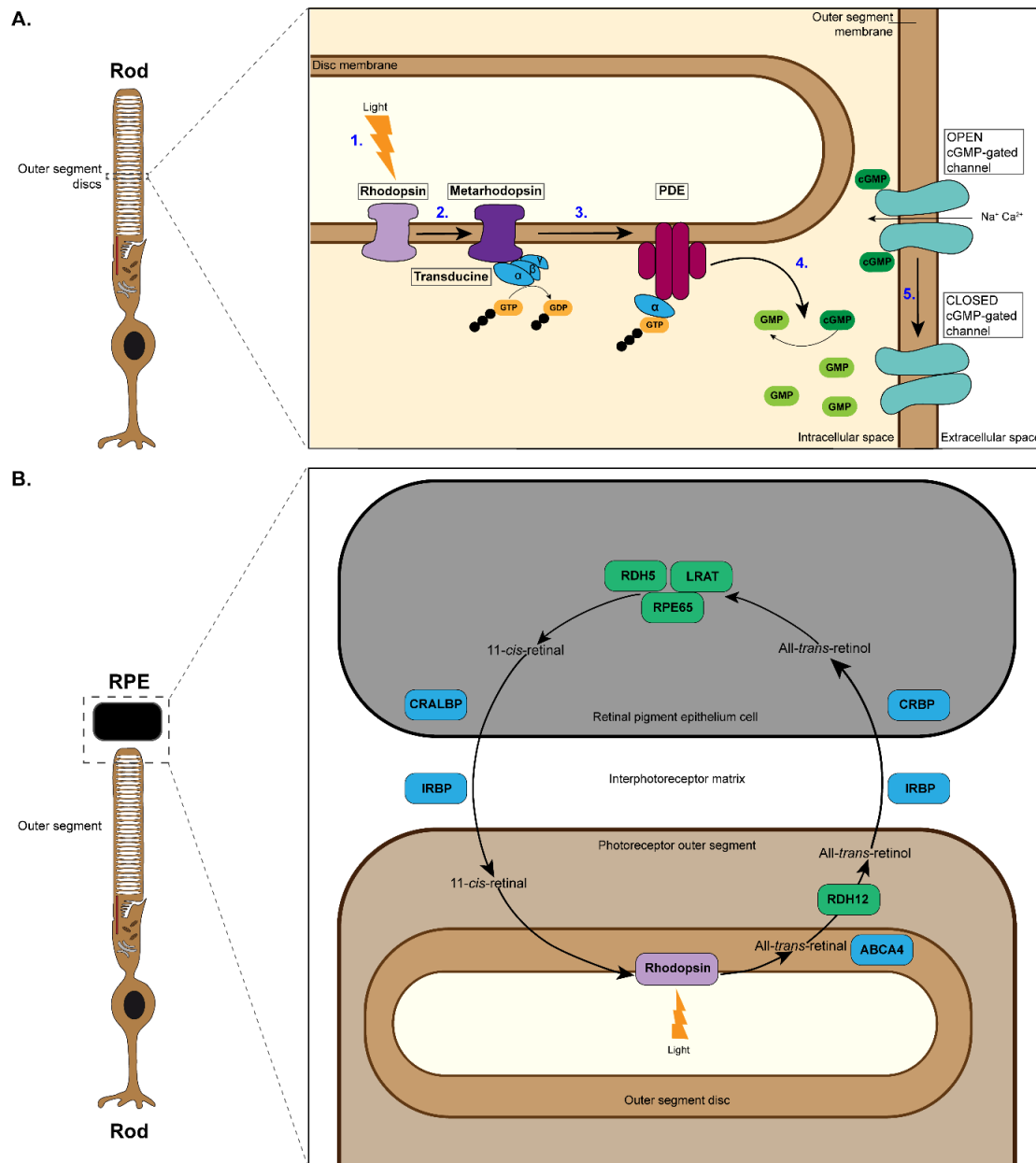


Figure 6 : Schematic representation of the phototransduction cascade and visual cycle in rods' apical region.

A/Phototransduction occurs in the outer segment discs within rod outer segment. In the dark, CNG channels are opened allowing cations influx. Light activates Rhodopsin in metarhodopsin (1.) that binds Transducin, activating its α subunit (2.). Then transducin activates Phosphodiesterase (PDE) (3.) and hydrolyzes cGMP into GMP (4.). cGMP is no longer available leading to cGMPgated channels close and thus leads to hyperpolarization of photoreceptors (5.). In the dark, CNG channels are opened and allow the cations influx and photoreceptors' depolarization.

B/ Visual cycle takes place in photoreceptor outer segments and in the RPE. Photon absorption leads to the conversion of 11-cis-retinal binds to Rhodopsin to all-trans-retinal. All-trans-retinal is carried out of the photoreceptors by the Interstitial Retinal Binding Protein (IRBP) transporter and re-isomerized to 11-cis-retinal in the RPE. Transporters (in blue): ABCA4, ATP-binding cassette subfamily A member 4; IRBP, Interstitial Retinal Binding Protein; CRBP, Cellular Retinol Binding Protein; CRALBP, Cellular Retinaldehyde Binding Protein. Enzymes (in green): RDH12, retinol dehydrogenase 12; LRAT, Lecithin Retinol Acyltransferase; RPE65, RPE 65 isomerase; RDH5, retinol dehydrogenase 5 (Gagliardi 2018).

3. Support cells in the retina

3.1 The Retinal Pigmentary Epithelium (RPE): a non-neuronal support cell

The RPE are pigmented cells that ensure light absorption by reducing backscattering of light that enters the eye. They play several key functions in the visual process with their phagocytosis and endocytosis capacities (Finnemann et al. 1997; Heth and Bernstein 1991). Located between the photoreceptors and the choroid, RPE cells are involved in the outer blood barrier of the retina and thus control the composition of the sub-retinal spaces providing metabolic support to retinal cells. They closely interact with the photoreceptor outer segments allowing the renewal of the outer segments through phagocytosis. Indeed, photoreceptors are prone to photo-oxidative damage and phagocytosis of aged portions of the outer segment is necessary for their long-term health. Moreover, they are involved in the visual cycle in the recycling of visual pigments.

3.2 Retinal Glial cells as support cells within the neural retina

Retinal glial cells are composed of astrocytes, microglia cells and Müller cells. Astrocytes are part of the blood retinal barrier located in the RGC layer. Microglial cells are part of the local immune system of the retina. Finally, Müller cells have a key role in the homeostasis, metabolic and architectural support, and neuroprotection of the retina.

4. Extracellular matrix

The extracellular matrix (ECM) or extracellular space (ECS) is composed of proteoglycans (heparan sulfate, chondroitin sulfate, and keratin sulfate), nonproteoglycan polysaccharides (hyaluronic acid), fibers (collagen and elastin), and other proteins such as fibronectin, fibrillin, laminins, and fibulins. Minor components of the ECM include growth factors, immune mediators such as complement system components (e.g., complement factor H and vitronectin) and extracellular proteases (e.g., matrix metalloproteinases [MMPs]). These components are secreted locally and assembled into a well-organized network associated with the surrounding cells. The extracellular matrix that fills the subretinal space is defined as the interphotoreceptor matrix (IPM).

ECM supports the organization and the functionality of retinal cells. Particular, roles of the IPM are; retinal adhesion to the RPE, retinoid transport, cytoskeleton arrangement and transport of oxygen and nutrients to the PRs. During early postnatal development, ECS volume fraction or spaces between cell bodies are larger compared to mature retina, specifically in the GCL and the INL (Fig) (Kuo et al. 2020) (**Figure 7**). Then changes in the ECM occur during the aging or diseases process (Al-Ubaidi et al. 2013). In retinal degeneration, loss of cells and thus of integrin receptors (attachment points between ECM and retinal cells) trigger a progressive structural disruption of ECM. These changes have an impact on remaining cells' functionality and the shuttling of retinoids between the retina and the RPE (Jackson et al. 2002).

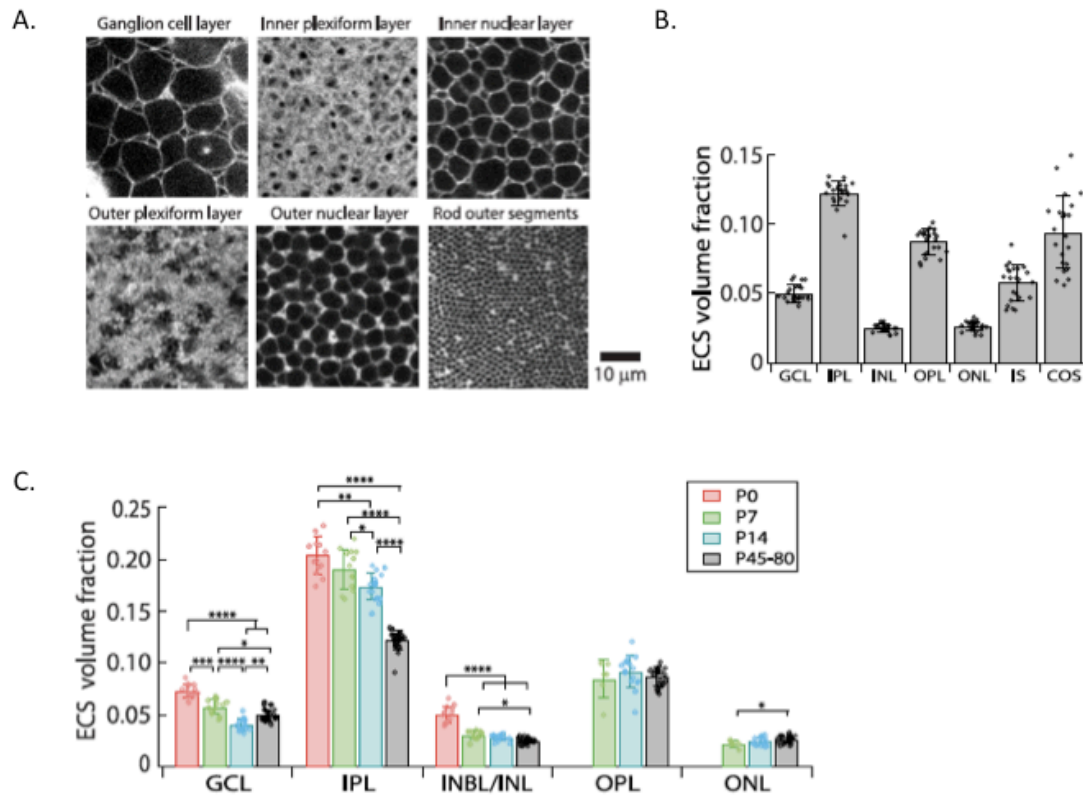


Figure 7 : Extracellular space (ECS) volume in different layers of the adult retina.

A/ Two-photon microscopy images of calcein fluorescence in the different layers of the retina. B/ Summary of ECS volume fraction in different retinal layers. C/ Summary of ECS volume fraction in different retinal layers across development. ECS in GCL and INL is wider in young retina at P0, P7 and P14 compared to mature retina. Adapted from (Kuo et al. 2020).

II. Retinal diseases and gene therapy strategies

Visual loss from outer retinal dystrophies is the major cause of blindness in industrialized countries (Wright et al. 2010). Whatever the cause, retinal degenerations ultimately result from the progressive loss of photoreceptors.

Inherited retinal dystrophies (IRD) are due to genetic mutations that photoreceptors carry or mutations found in RPE cells (Mendes et al. 2005). Retinal dystrophies can be also complex diseases acquired later in life and be caused by a combination of both genetic and environmental factors.

1. Genetic retinal diseases

In developed countries, genetic causes account for over 50% of all cases of vision loss. The individual mutations or causal variants of each of the sensory defective genes are relatively rare, but, taken together, they are a significant cause of blindness, particularly in individuals of working age, which increases their economic and societal impact. Inherited blindness is clinically and genetically heterogeneous. Retinal dystrophies are often characterized by progressive degeneration of the neural retina and/or the RPE. More than 300 genes have been implicated in inherited vision impairments (RetNet: <https://sph.uth.edu/RetNet/>). Those genes are involved in numerous and diverse cell and tissue functions. Clinically, the forms of inherited retinal degeneration vary from early-onset macular degeneration, a progressive type of degeneration affecting central vision, to retinitis pigmentosa (RP) and Leber congenital amaurosis (LCA), which often manifest at birth and initially involves rod photoreceptors at the periphery of the retina. Inherited retinal degeneration may display autosomal-dominant, autosomal-recessive, or X-linked inheritance, and more complex or multifactorial inheritance patterns have also been reported, especially for some of the later-onset progressive diseases.

1.1 Retinitis pigmentosa, the most common form of IRD

Retinitis pigmentosa (RP) is the broadest category of IRD and represents 40% of IRD cases. RP can start from early to middle adulthood and disease progression is variable. A large number of genes are involved. The most frequent mutations are found on the rhodopsin gene and lead either to overexpression or under-expression of the rhodopsin protein or the presence of unfolded proteins in the endoplasmic reticulum (Kunte et al. 2012).

Despite the genetic complexity and heterogeneity of RP, there is a common phenotype. Here, we divided RP progression in 4 stages depending on where and which cell types remain in the retina (**Figure 8**).

1) Rod photoreceptors start to degenerate from the periphery towards the center of the retina as a consequence of the mutation (B. Lin et al. 2009). The degeneration of rods involves apoptosis (Cottet and Schorderet 2009), however, the mechanism leading to cell death has still not been entirely elucidated for every mutation. Previous studies suggest that the accumulation of unfolded or misfolded protein can be toxic for the rod photoreceptors (J. H. Lin and Lavail 2010). Other studies suggest microglia and oxidative stress may also contribute (Di Pierdomenico et al. 2019; Zeng et al. 2014) and mutations in the interphotoreceptor retinoid-binding protein have also been reported to activate apoptotic and necrotic pathways (Sato et al. 2013). Regardless of the mutation, rod degeneration results in night blindness, that is, the loss of ability to see in the dark or low-lit environments. Night blindness is usually the first symptom of rod-cone dystrophies (RCD) and addressing the disease at this point is challenging, requiring knowledge of the mutation and means to repair it or replace it before major loss of rods.

2) In the second stage of the degeneration, the loss of rods causes the degeneration of cone outer segments from the periphery towards the center of the retina. Rods are responsible for the production of rod-derived cone viability factor (RdCVF), a trophic factor that is fundamental for cone survival (Léveillard et al. 2004). The result of cone outer segment loss is a progressive shrinking of the visual field, resulting in “tunnel vision” (**Figure 9**).

3) In the third stage of RCD, surviving cone cells lose their light responsive outer segment. Indeed the maintenance of the outer segment is metabolically demanding and difficult to maintain in absence of trophic support from rods. At this stage, remaining cones (mostly in the fovea) become sparser and lose their outer segment leading to loss of the remaining tunnel vision.

4) In the fourth and final, stage of degeneration, cone cell bodies might completely degenerate, leaving the retina with only inner retinal neurons (Stone et al. 1992).

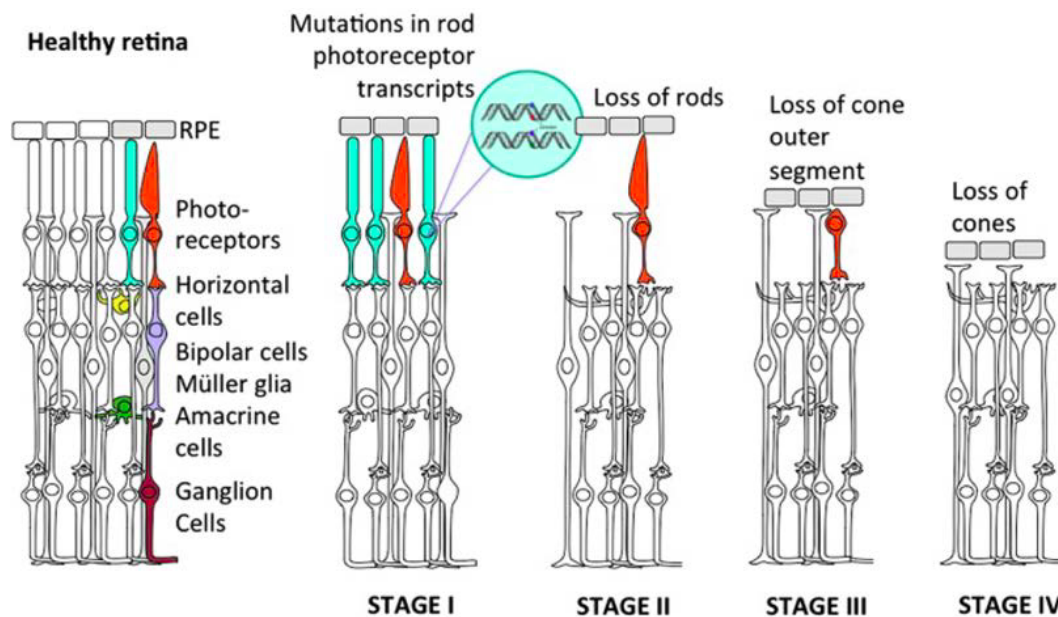


Figure 8 : Timecourse of cell death and vision loss in Retinitis pigmentosa (RP).

First, mutations mostly in rod transcripts lead to degeneration of these cells (stage I) followed by progressive cone degeneration that spans two distinct stages (stages II and III). In stage II, cones still have an outer and inner segment, whereas these features are lost in the subsequent stage III, leading to so-called "dormant cones". In the final stage (stage IV), all photoreceptor cells are lost altogether. (Dalkara et al. 2016)

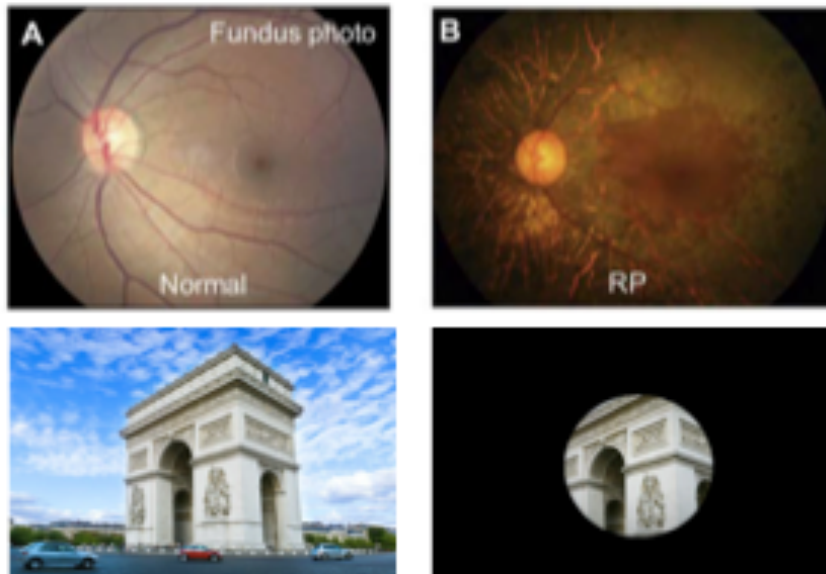


Figure 9 : Retinitis pigmentosa (RP) phenotypic and visual comparison.

A/ Eye fundus of a healthy retina correlated to daylight vision. B/ Eye fundus of mid-stage RP patient retinas with presence of retinal deposits and abnormal vessels, except in the macula. RP leads first to night and peripheral blindness with visual field restriction due to rod photoreceptors' death. Then, secondary degeneration of cones leads to visual field constriction. At this point, only macula remains leading to tunnel vision. Adapted from (Dias et al. 2018).

1.2 Usher syndrome

Along my Ph.D. training, I focused on Usher syndrome type 1B. I developed a transient delivery method for gene editing tools with the long-term goal to apply it to Usher syndrome. Therefore, a schematic overview on auditory and visual impairments due to this disease is presented to help the readers better understand long-term goals of my research.

Usher syndrome is an autosomal recessive disease with a prevalence estimated to be 1/6000 (Kimberling et al. 2010). There are three clinical subtypes based on the RP onset and the degree of hearing loss. Among them, USH type 1 is the most severe one with congenital severe to profound hearing loss, a pre-pubertal RP onset associated with a vestibular dysfunction.

Usher syndrome is genetically heterogeneous with thirteen identified genes, all of which code for usher proteins (Bonnet and El-Amraoui 2012). Among these genes, the first discovered was a 7kb gene encoding for MYO7a protein. Several mutations along this gene have been reported to lead to USH type 1 B disease (USH1B).

Usher proteins are organized in a protein interactome in both the inner ear (hair bundles and synapse) and the retina (calyceal processes and synapse of the photoreceptors). They have various functions including actin-based intracellular trafficking, cell adhesion, scaffolds in multiprotein complexes, and G protein- or Ca^{2+} -mediated signaling. Mutation in one of this protein would compromise all the complexes' function. However, the lack of retinal phenotype in mice model with no retinal degeneration in case of Ush1B prevent to understand the mechanisms underlying the retinal degeneration in this disease (El-Amraoui and Petit 2014). This lack of phenotype would be due to the absence of calyceal processes in this rodent model (El-Amraoui and Petit 2014; I. Sahly et al. 2012a).

There is no existing curative treatment for Usher diseases. Indeed, current treatments for hearing loss are limited to hearing aids and cochlear implants, which partially restore hearing abilities (Kleinlogel et al. 2020; Müller and Barr-Gillespie 2015). Gene therapy is a promising strategy to treat Usher syndrome. Some strategies are already in clinics and will be discussed in the gene replacement therapy part.

2. Complex photoreceptor degenerations

The other large group of diseases affecting photoreceptor survival is multifactorial outer retinal diseases caused by environmental factors in addition to proven genetic risk factors. The most common multifactorial disease is age-related macular degeneration (AMD). First cause of blindness in people over 50 years old, it is estimated that the number of people affected by AMD will increase to almost 300 million in 2040 (Wong et al. 2014).

AMD pathogenesis is characterized by the presence in the macula of lipids deposits, named drusen, between the RPE and an adjacent basement membrane complex known as Bruch's membrane. Drusen are accompanied with chronic inflammation, oxidative stress and impaired extracellular matrix maintenance. With advancing disease, progressive degeneration of neural retina and RPE cells causes loss of central vision (Fleckenstein et al. 2021). In contrast to targeted replacement of single gene deficiencies that can be proposed in monogenic diseases described above, different strategies are required to treat these so-called complex photoreceptor degenerations, like AMD.

3. Gene therapy strategies dependent on the stage of the diseases

The eye is particularly a suitable organ for a wide range of therapeutic interventions. Indeed, there is a minimal risk of therapeutic agents diffusing to unwanted tissues or organs upon local delivery, due to the confined environment of the compartments concerned. In addition, this organ is a unique zone of immune privilege, thanks to a blood-organ barrier impeding movement of cells and molecules into and out of the sensory epithelia (Bucher et al. 2021).

In addition to that, progress in high-resolution retinal imaging for clinical investigations (Rossi et al. 2011) and increased efforts have been devoted over the past years to improve early genetic diagnosis for blindness disorders (de Bruijn et al. 2021; Thompson et al. 2020). In parallel, understanding of the genetics and pathophysiological mechanisms has been improved. Together these advancements lead to develop treatment solutions to restore normal gene function by retinal gene therapy (Kleinlogel et al. 2020; Maguire et al. 2021).

The primordial question in moving towards successful implementation of therapies is matching the patient genotype/phenotype at the time of intervention with an adequate therapeutic approach. For simplification, we will focus on the predominant phenotype encountered in rod-cone dystrophy patients. As mentioned above below we discuss different therapies in view of their utility in each stage and in some stationary diseases that fall outside these phenotypic manifestations.

In principal, the most significant benefit from gene therapy can be obtained by replacing the mutant gene with a healthy copy or by correcting the underlying mutation prior to degeneration of retinal cells. Some progressive diseases have a large window of opportunity where such interventions can be made prior to the loss of photoreceptors resulting in potentially lifetime benefit. However, in most cases of rod-cone dystrophy, once photoreceptors have started to degenerate, gene replacement or correction is uncertain to provide a life-long benefit, as the degenerative process once underway may be difficult to

halt. Moreover, there are many unknown and dominant mutations where gene replacement cannot be envisioned.

3.1 Stage 1: Gene replacement strategy

The first applications of gene therapy were focused on gene replacement (also called gene addition or gene supplementation) therapy that consists to replace the mutant gene with a healthy copy prior to degeneration of retinal cells. Over 2 decades, at least five different adeno-associated virus (AAV)-RPE65 products were tested using mouse and canine models of Leber congenital amaurosis 2 (LCA2), which led to a total of 13 clinical trials (Maguire et al. 2021). This pioneering work led to the historical approval of the first gene therapy product, Luxturna®, by the Food and Drug Administration (FDA) and European Medicines Agency (EMA) to treat Leber congenital amaurosis. This groundbreaking advancement of *RPE65* gene therapy paved the way for numerous breakthrough clinical phase I/II trials exploring gene replacement for monogenic recessive diseases of the retina, such as choroideremia, retinoschisis, achromatopsia, Usher syndrome, and Leber hereditary optic neuropathy (Botto et al. 2022; Buck and Wijnholds 2020).

Gene replacement for monogenic recessive diseases

After an intense period of gene identification in the 80s and 90s, it became evident that some of the monogenic recessive diseases could benefit from the so-called gene replacement approach.

With the advances in vector technology (see part IV) encouraging results started to be obtained in model animals in several retinal dystrophies. Among those, type 2 LCA seemed to be an excellent candidate as the causal gene *RPE65* had been identified and was associated with a slowly progressing phenotype where the retina stayed in stages II and III (Schimmer and Breazzano 2015). Photoreceptors persisted over decades leading to a rather large window of opportunity for therapeutic intervention. Mutations in the gene encoding the RPE specific protein RPE65 account for about 5–10% of LCA cases (Apte 2018; Thompson et al. 2000). Encouraging results from both small and large animal showed that AAV-mediated RPE65 expression slowed down or reverse vision loss, and paved the way toward first application in humans (Bennett 2017). Adult patients of various ages received a single

subretinal injection of AAV vector carrying the *RPE65* gene in their worst-seeing eye leading showing both the safety and efficacy of this approach (Bainbridge and Ali 2008). The improvements in scores of best-corrected visual acuity, kinetic visual field, nystagmus, pupillary light reflex, microperimetry, dark-adapted perimetry, dark-adapted full-field sensitivity in treated eyes compared to untreated eyes confirmed the benefits and paved the way for administration of AAV-RPE65 to contralateral eyes of some patients where recovery of retinal function was also observed in the second eye. However, two groups reported that early visual improvements in RPE65-treated patients with LCA were limited in time in some patients. In a group of patients, visual benefits persisted up to 3 years, but two years later, the areas of improved vision (visual field) were found to have constricted (Bainbridge et al. 2015; Jacobson et al. 2015). This indicated that in adults, the degeneration of photoreceptors continued despite the initial improvement in function. Continued degeneration in treated retina can be due to the insufficient amount of RPE65 provided by the therapy or can be mediated by progressive loss of trophic support (particularly for cones) (Aït-Ali et al. 2015). The possibility that the rods had progressed to a pre-apoptotic state by the time the intervention could also be an explanation to the limited duration of therapeutic benefit in these studies. The loss of visual function at later times after treatment is in line with this natural progression of degeneration. After this first application, gene replacement therapy was quickly implemented for other retinal degenerative diseases based on the promising outcomes of these pioneering studies. Gene replacement therapy for choroideremia, and other forms of LCA caused by mutations in various genes (i.e. *GUCY2D*), X-linked retinoschisis (XLRS), *MERTK*, achromatopsia came quickly as these therapies build on the successful application of AAV vectors in the subretinal space to encode replacement genes in the outer retina. Leber hereditary optic neuropathy (LHON) - a maternally inherited disease caused by mitochondrial DNA mutations also implemented use of AAVs for gene replacement via intravitreal AAV delivery with successful outcomes and new findings specific to this disease where the optic nerve implications showed unanticipated results (Dalkara et al. 2016). Other studies used lentiviral vectors as cDNA copies of mutant genes were too large to be carried by AAV. Clinical trials to treat Stargardt disease (STGD) associated with mutations in the photoreceptor-specific *ABCA4* gene and *Myo7A* associated Usher Syndrome are two examples. Although no serious adverse events related to the vector have been reported so far, efficacy in these diseases might be limited by the tropism of lentiviral

vectors (see in part IV). Preliminary studies towards the development of gene therapy for CEP290-associated LCA (LCA10) were also conducted. LV vector expressing full-length human CEP290 were to be used but due to doubts about photoreceptor transduction using LV, other approaches were prioritized such as mutation read through with antisense oligonucleotides (ASOs) or by CRISPR/Cas9-mediated gene editing (DiCarlo et al. 2018). Both of these technologies came into spotlight as a potential solution to the limited length of replacement genes that can be delivered with AAV vectors (see in part IV).

Silence and replace for dominant diseases

For patients in Stage I, gene replacement is technologically the most straightforward option for reversing the course of recessive retinal diseases caused by loss-of-function mutations. Unfortunately, this technique is not suitable for autosomal dominant (ad) diseases. Indeed, in this type of disease, the gene therapy approach will depend on the type of mutation. Autosomal dominant diseases can be caused by dominant-negative or gain-of-function mutations leading to proteins with antagonistic effect to the wild-type one in case of dominant-negative mutations. In gain-of-function mutations, the encoded protein has a new function, also leading to toxicity. Therefore, on these types of mutations, a simple healthy gene supplementation is not enough: the mutated gene needs to be silenced to inactivate the detrimental effect (gene silencing). Depending on the disease and on its stage, an additional supplementation might be necessary (“silence and replace”) (Farrar et al. 2012). The other gene therapy options for Stage I diseases caused by dominant-negative or gain-of-function mutations is gene correction as discussed further below (See in part V).

- Gene silencing

Autosomal dominant Retinitis Pigmentosa (adRP) accounts for around 30% of all RP cases and among adRP, ~25% are due to mutations in the rhodopsin *RHO* gene. No gene therapy applicable in Stage I of adRP has reached the clinic so far due to the more complex nature of substituting a healthy *RHO* gene while silencing the mutant copy. The main target of genetic silencing strategies is the messenger RNA (mRNA) transcript, the function of which can be inhibited by antisense RNA-based, ribozyme-based and more recently by small interfering (si) RNA-based and micro (mi) RNA-based, approaches. In view of the allelic heterogeneity of the *RHO* mutations, gene-silencing approaches, using ribozyme or RNA interference, have

been developed to suppress both the wild type and mutant rhodopsin. Such approaches have the advantage of being applicable to multiple mutations. For example, Jiang and colleagues developed an shRNA that significantly improved photoreceptor survival, delayed disease onset, and ameliorated visual function in an adRP mouse model expressing bovine GCAP1 (Y99C) (L. Jiang et al. 2011). But *RHO* is an essential gene for the retinal function and its complete suppression will induce phenotypic degradation, as it is the case in recessive RP caused by *RHO* mutations. Therefore, mutation-independent suppression of both alleles will probably require supplementation by the addition of exogenous rhodopsin in order to achieve optimal therapeutic benefit.

- Gene replacement after silencing

In an interesting past study, some benefit in slowing down the loss of rods had been obtained when *RHO* expression alone was achieved in a mouse model of adRP (Mao et al. 2011). Contrarily, in a more recent attempt of gene replacement alone in a mouse model *RHO*. P23H, no beneficial effect on retinal structure or function was observed (Orlans et al. 2020). Therefore, more efforts have been focused on coupling the silencing and the replacement. As recent studies also suggest, in *RHO* replacement it is important to monitor closely protein levels, depending on the model, as a too small quantity of protein will not be sufficient to obtain a significant rescue but an excess of expression might be toxic to the cell. For example, an excess of *RHO* expression level (151%) in control mice leads to outer retina toxicity (Orlans et al. 2020). it has been developed for silencing alone, several groups used RNA interference techniques to silence *RHO*, to which they add a replacement of *RHO* cDNA. They all did subretinal injection at early stage (from D1 to D14). In 2007, a study used a single AAV to deliver siRNA and shRNA simultaneously targeting the *RHO* cDNA that leads to an increased ONL thickness (O'Reilly et al. 2007). Another study confirmed these results but with the use of 2 AAVs and with an additional increase of the scotopic ERG at 20-weeks post-injection (Millington-Ward et al. 2011). The persistence of the preserved ERG response and ONL thickness was studied up to 9 months post-injection in an additional study (Mao et al. 2012) as a follow up to their demonstrated therapeutic benefits of *RHO* gene expression in absence of silencing (Mao et al. 2011). A possible alternative to such RNA-targeting approaches is the modulation of gene expression at the transcriptional level, by using zinc-finger (ZF)- based artificial transcription factors (ZF-ATFs) that can be tailored to a desired

DNA target sequence (Stolzenburg et al. 2010). In a newly designed two-step repression–replacement strategy mutations leading adRP has been targeted with zinc-fingers, also in combination with a replacement of *RHO* gene, showing respectively, a preserved ERG response at 60 days post-injection and the integrity of the photoreceptor outer segments (Botta et al. 2016; Mussolino et al. 2011). Recent translation of silence and replace strategy a large animal model of adRP showed that the development of clinical therapies needs to fine-tune the level of reduction of both mutant and WT endogenous proteins while providing sufficient resistant replacement (Cideciyan et al. 2018) with a particular focus on improving *RHO* supplementation. The discovery and ease of application of CRISPR (see below) has lent its use as a mutation-independent gene silencer instead of RNA interference and will be discussed in part V.

3.2 Stage 2 and 3: Neuroprotection and optogenetics

In early phases of stage 2, rod numbers have decreased but central cones are still viable and patients retain central vision, a therapeutic approach should aim at (1) slowing down photoreceptor degeneration, (2) preserving cone function in the fovea. One way to address these two points is by providing the retina with neuroprotection (Wubben et al. 2019). Neurotrophic factors are small molecules, mostly peptides. They promote cell growth, proliferation, differentiation and survival with either an autocrine or a paracrine effect (Kolomeyer and Zarbin 2014). The identification of one mechanism that causes vision loss in rod-cone dystrophies revealed a signaling molecule called Rod-derived cone viability factor (RdCVF) that represents a promising therapy specific for this disease mechanism (Léveillard and Sahel 2010). RdCVF, maintains the function and the viability of cone photoreceptor cells in the retina by stimulating glycolysis; and mice that lack this factor exhibit a progressive loss of photoreceptor cells (Aït-Ali et al. 2015). The gene encoding RdCVF also encodes, by differential splicing, a second product that has characteristics of a thioredoxin-like enzyme and protects both rod and cone photoreceptor cells against oxidative damage via its interacting protein partner, the tau protein (Byrne et al. 2015; Mei et al. 2016). Expression of RdCVF has been proven beneficial in several animal models, and an RdCVF-based gene therapy is currently being developed for clinical application.

As the degeneration progresses, however, cone outer segments are lost in the central retina as well, and optogenetic approaches aimed at restoring light sensitivity in dormant cones can be envisioned. Since in healthy retinas photoreceptors hyperpolarize in response to a light stimulus, the idea is to provide remaining cones with microbial opsins capable of hyperpolarizing the membrane without the need for the intricate phototransduction cascade occurring in the outer segment. Indeed, once light capture has been lost, it is interesting to observe that the inner retinal neural circuitry from bipolar cells to retinal ganglion cells (RGCs) often remain intact. This gives rise to further opportunities to salvage vision in advanced disease via the use of gene therapy.

3.3 Stage 4: Optogenetics

In the last stage of retinal degeneration, almost the entirety of photoreceptors might be lost, in which case the RPE invades the subretinal space and the inner retina undergoes remodeling and rewiring (Marc and Jones 2003). In this scenario, a different group of therapies aiming to compensate photoreceptor function are used. Optogenetics is once again implemented here but this time to generate currents in normally light-insensitive second and third order neurons (Kleinlogel et al. 2020; Scholl et al. 2016) promising success to restore sense perception in ongoing clinical trials (Sahel et al. 2021).

III. CRISPR-Cas gene editing

The CRISPR Cas system, which stands for Clustered Regularly Interspaced Short Palindromic Repeats associated protein Cas, represents a breakthrough in the field of genetics and gene therapy. CRISPR Cas, often compared to a genetic scissors, allows targeting and then cutting any sequence in the genome with high precision. Discovered through the study of microorganisms, the CRISPR Cas system has evolved at a very high speed to become the simplest, most versatile and least expensive editing tool.

Clinical trials of gene editing tools are already underway for several specific human conditions, opening new possibilities for human genetic and/or epigenetic disorders based on gene and RNA editing (Anzalone et al. 2020).

1. CRISPR Cas system: origin, mechanisms and limitations

1.1 The bacterial origin of CRISPR and its adaptation to mammalian systems

Prokaryotes undergo numerous viral attacks by bacteriophages. These attacks have resulted in a very strong selective pressure leading to the development of adaptive immunity defense mechanisms named CRISPR Cas (**Figure 10**).

The entry of foreign genetic elements into bacteria, such as during a viral or plasmid attack, will trigger this system through three steps: adaptation, expression and interference. (Deveau et al. 2010; van der Oost et al. 2009). The adaptation step allows the acquisition of short DNA sequences called spacers that are homologous to viral or plasmid sequences called protospacers. The selection of protospacers in the foreign DNA will be determined by the presence of short sequences of a few nucleotides called PAM. The PAM sequence varies depending on the Cas9 protein used. (Mojica et al. 2009). These spacers are then integrated into CRISPR loci by proteins (Cas1 and Cas2) that are highly conserved between different CRISPR systems. Each integration is accompanied by the duplication of a repeat within the CRISPR locus to create a new spacer-repeat unit. The acquisition of this new spacer will confer on the bacterium a resistance to the microorganism from which it originated.

The second step of this bacterial immunity system is the expression of a long primary transcript of the CRISPR locus called pre-crRNA. An endonuclease will catalyze the cleavage of this pre-crRNA into short crRNAs. In *S. pyogenes*, the pre-crRNA is catalyzed by RNase III in the presence of Cas9 and a tracrRNA that guides maturation.

In a second attack, the foreign DNA will be recognized by the crRNA that will guide the different protein complexes by pairing with the complementary sequence of the proto-spacer. The protein complexes will be able to cleave this sequence and thus destroy the foreign DNA. This process, called interference, is only allowed at the level of DNA sequences containing a PAM.

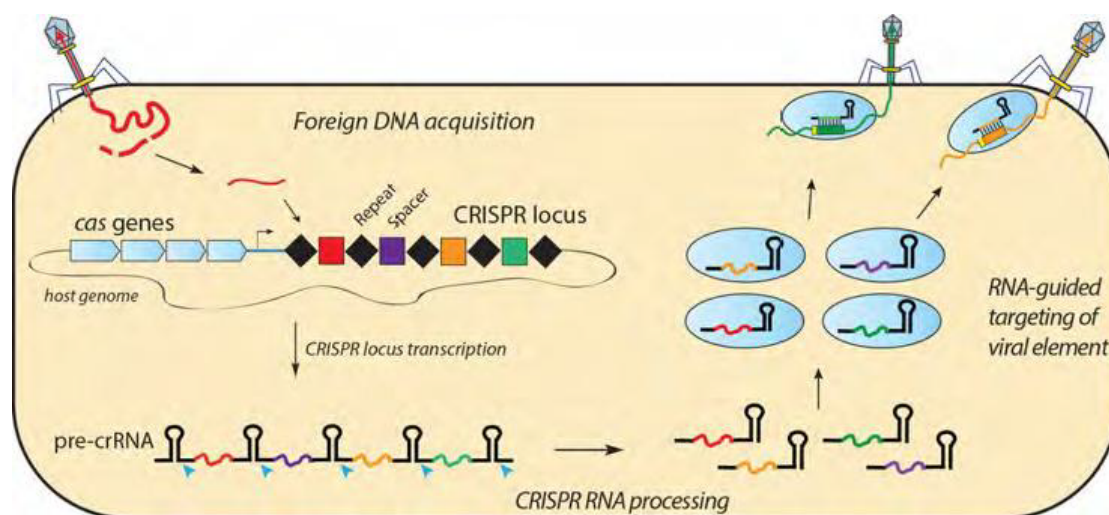


Figure 10 : CRISPR system in prokaryotes, a defense mechanism of adaptive immunity.

During a first viral attack, the adaptation step will allow the acquisition of short viral DNA sequences, the protospacers. These sequences are then integrated into the CRISPR loci to form a spacer-repeat unit and will allow the expression of a pre-crRNA that will be subsequently matured. In a second viral attack, the associated crRNAs of the protein complexes will allow cleavage of the viral genome. Illustration from (<http://rna.berkeley.edu/crispr.html>)

The better understanding of the mechanism of action of CRISPR Cas as a bacterial immunity system has allowed to reveal the full potential of this system and to consider its hijacking into one of the most powerful genome editing tools.

The CRISPR type II system is the simplest system since it requires the use of a single nuclease, such as Cas9, contrary to other systems. It was therefore possible to adapt it for genome editing in mammalian cells. The first step was to further simplify it by linking the tracrRNA sequence and the crRNA with a loop to create a single gRNA called single guide RNA (sgRNA). This modification reported in 2012 by the teams of J.Doudna and E.Charpentier has greatly facilitated the use of the CRISPR Cas system as a genome editing tool (Jinek et al. 2012).

1.2 The actors

Cas/sgRNA system is a ribonucleoprotein (RNP) complex formed by a Cas protein and a guide RNA (**Figure 11A**).

The single guide RNA is a small RNA of around 120 base pairs and composes of two sequences:

- at the 3' end, a tertiary double-stranded structure that allows binding to the Cas protein, called the scaffold.
 - at the 5' end, a sequence of 20 nucleotides complementary to the target DNA sequence.
- The gRNA is designed to identify and bind to a specific sequence in the DNA. Any sequence in the genome can be targeted as long as it is adjacent to a short sequence (on average 3 to 5 nucleotides) called the Protospacer Adjacent Motif or PAM sequence.

The scaffold RNA binds to the Cas protein and the predesigned sequence “guides” the enzyme to the part of the genome targeted for modification (**Figure 11A**). The Cas follows the gRNA to the target site in the DNA sequence, at which it cuts both strands of the DNA right upstream of the protospacer adjacent motif (PAM) in the genome thanks to its two endonuclease domains: RuvC at the N terminus and HNH near the center of the protein. (**Figure 13A1**). Unlike the PAM proximal blunt-end cuts generated by Cas9, Cas12 nucleases typically generate staggered cuts within regions of the protospacer that are distal to the PAM sequence, which is advantageous for applications such as integrating DNA fragments in a preferred orientation (Anzalone et al. 2020).

To increase editing specificity and to widen the scope of sequence recognition through various PAM motifs, dozens of Cas orthologs were discovered and engineered (e.g.,

SmaCas9, SaCas9, SaCas9-KKH, FnCas9-RHA, enAsCas12a, LbCas12a and AsCas12a, . . .; (Anzalone et al. 2020; Saha et al. 2021; Yu and Wu 2021) for a more exhaustive list).

X-ray crystallography and electron microscopy have provided insight into the structure and mechanism of action of the CRISPR Cas9 system (Dong et al. 2016; Jinek et al. 2014; Nishimasu et al. 2015).

The Cas9 protein consists of two lobes, the nuclease lobe and the alpha helix lobe, which are linked by an arginine-rich helix. It is at the level of the nuclease lobe that the two nuclease domains HNH and RuvC are located and also the arginine-rich C-terminal domain or CTD that permits binding to the PAM (**Figure 11B**). The Cas9 protein has two grooves on its molecular surface, composed mainly of positively charged amino acids (**Figure 11B**). After conformational rearrangement, its grooves form a canal that allows the binding of the gRNA and the targeted DNA, both negatively charged.

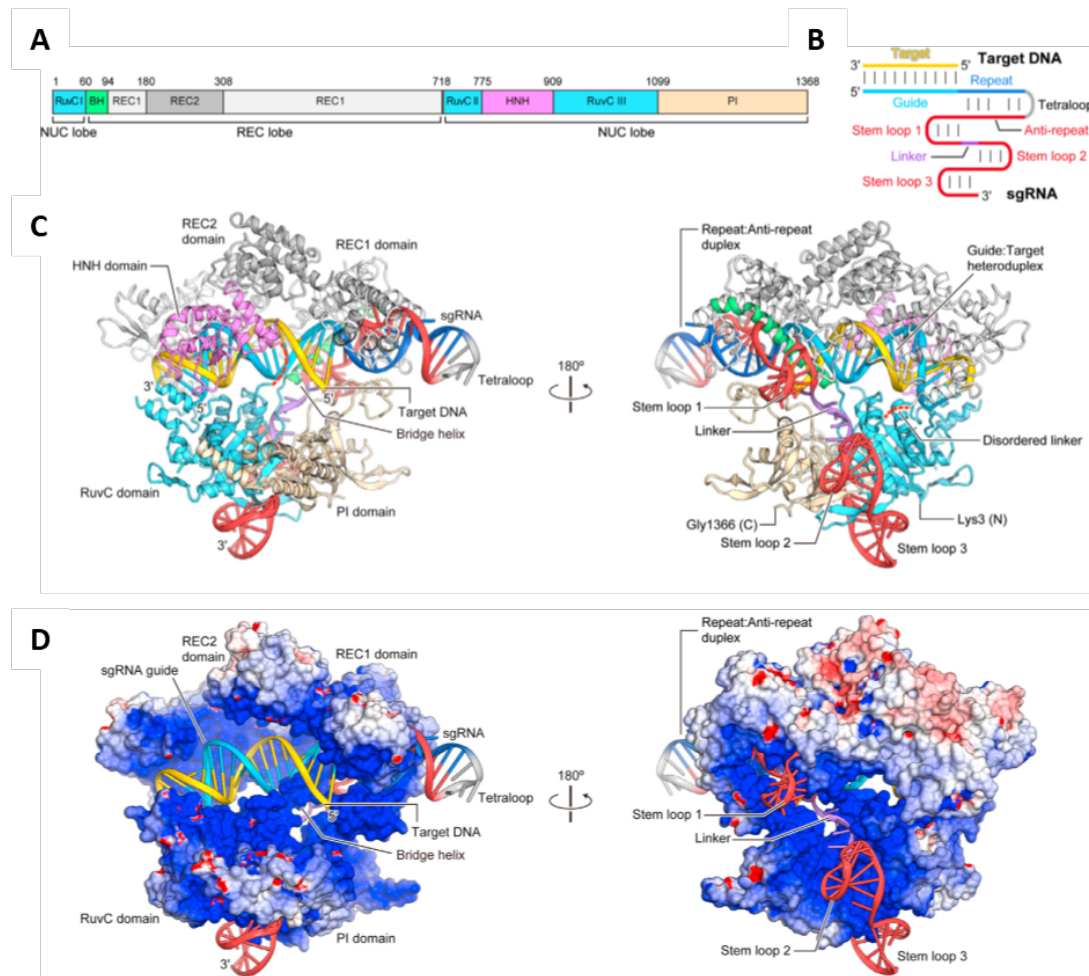


Figure 11 : Cas/sgRNA system, a genome editing tool.

A/ Domain organization of S. pyogenes Cas9. BH, bridge helix. B/Schematic representation of the sgRNA:target DNA complex. The gRNA is composed of a constant part recognizing the protein, in red, and a 20 bp sequence, in light blue, that can be modified to recognize any genome sequence as long as it is adjacent to the PAM sequence. C/ Ribbon representation of the Cas9-sgRNA-DNA complex. D/Electrostatic surface potential of Cas9 ranging from -10kT/e (red) to +10kT/e (blue). Adapted from (Nishimasu et al. 2014).

1.3 Molecular mechanisms of repair

The damaged DNA undergoes repair, predominantly by mechanisms naturally present in mammalian cells: mainly, the non-homologous end joining repair (NHEJ) resulting in insertions and deletions of nucleotides (referred to as indels) (**Figure 12**). In the presence of a donor DNA template (e.g., single-stranded oligonucleotide donors ssODNs), a homology-directed repair (HDR) mechanism can occur, mostly in dividing cells. This competing (typically less efficient) pathway is used to correct targeted mutations or to knock in larger DNA sequences. However, the efficiency of HDR following CRISPR-Cas mediated double-strand DNA breaks (DSBs) *in vivo* remains low.

Indeed, the cell cycle plays an important role in the choice of the repair pathway used by the cell. The NHEJ pathway is predominant during G1, S and G2 phases while the HDR pathway is limited to the end of S phase and in G2. The HDR pathway will therefore be used in a minor way and in dividing cells when sister chromatids can serve as a template for repair of newly replicated chromosomes. (S. Lin et al. 2014). HDR low efficiency has fostered searches for other strategies for improving DNA repair (**Figure 13A2–3**) (Levy et al. 2020). Strategies to enhance one specific pathway are currently developed (Liu et al. 2018). Proteins involved in HDR repair pathway are fused to Cas9 protein to promote this repair mechanism, however applications *in vivo* have not been reported yet.

A third repair pathway named microhomology-mediated end-joining (MMEJ) pathway and is highly active during M phase and early S phase of the cell cycle. Other minority mechanisms exist, such as Nucleotide Excision Repair (NER) and Base Excision Repair (BER), which modify a single strand of DNA (Fortini and Dogliotti 2007). However, not all the repair mechanisms that can occur after a double or single stranded cut are well known (Baiken et al. 2020).

Note that these mechanisms can have different efficiency depending on the cell type.



Figure 12 : The NHEJ, HDR and MMEJ repair pathways with the different known proteins involved in each repair.

DSB is recognized and bound by ATM (Ataxia telangiectasia mutated) and ATR (Ataxia telangiectasia and Rad3-related protein) leading to the phosphorylation of H2AX in γH2AX. H2AX belongs to histone families organizing the eukaryotic DNA into chromatin and it is used as a DSB marker. The ubiquitinylation of γH2AX results in the recruitment of early phase DNA repair proteins, amongst which is p53 binding protein 1 (53bp1). 53bp1 recruitment triggers the NHEJ repair pathway and inhibition of the HDR pathway. Adapted From (Yanik et al. 2017)

Efficiency of reparation in the retina cells

In vivo DNA repair mechanisms are not well known in post-mitotic neurons such as photoreceptors. One study shows that especially rods have a low DSB repair efficiency *in vivo* after irradiation, a defect that is not observed in any other cell type of the adult retina. In this study, Frohns and colleagues reported that rods of adult wild type mice are deficient in repair pathway involving ATM protein. This deficit is found in adult nocturnal and diurnal animals. However, human rods or developing mouse retina have shown a higher DNA repair activity (Frohns et al. 2020). More studies are needed to understand DSB responses in the retina in each model used for gene therapy development.

1.4 Off targets

Complementarity between the gRNA and the target sequence is required for Cas9 activation. However, sequences partially complementary to the gRNA and located downstream of a PAM sequence can result in off-target cuts. It has been shown that the first 10 to 12 bases of the gRNA from the 5' region of the PAM are the most important for Cas9 activation. Cas9 can accept 1 to 6 mismatches. Subsequently, off-target cuts will be preferentially corrected by the introduction of indels via the NHEJ pathway, which will be mostly deleterious with a shift in the reading frame until the gene is inactivated.

Several factors may play a role in these undesired effects. According to O'Geen et al. 2015, gRNA is mostly responsible for the specificity of the system with respect to the protein Cas9 (O'Geen et al. 2015). In addition, chromatin accessibility may play into unwanted cuts. Because unmethylated regions are more accessible, they will be more likely to be modified (Wu et al. 2014).

1.5 Toxicity and immune responses related to gene editing tools

Toxicity due to double stranded breaks induced by Cas9/sqRNA

The double strand break induced by Cas leads to cellular toxicity. Indeed, during a stress such as a DNA lesion, an activation phase leads to an increase in the amount of p53 in the cell. This will lead to either cell cycle arrest in order to stimulate repair or cell apoptosis (Haapaniemi et al. 2018). New technologies of base editing and prime editing bypass double strand cuts to edit the genome and could prevent p53 activation (see below).

Immune reactions related to Cas9 proteins

The SpCas9 and SaCas9 proteins, commonly used in genome editing, are derived from *Staphylococcus aureus* (*S. aureus*) and *Streptococcus pyogenes* (*S. pyogenes*), respectively. They are common human commensal bacteria that are present on body surfaces covered by epithelial cells and are exposed to the external environment under normal condition. However, they are able to overcome protective host responses and be pathogenic in certain cases.

An epidemiological study has shown that 40% of the world's population is colonized by *S. aureus* (Lowy 1998) and 20% of the child population is colonized by *S. pyogenes* (Roberts et al. 2012). Further studies of the humoral and cellular adaptive responses against these two bacteria have been made. The results show a pre-existing humoral response of 79% for SaCas9 and 65% for SpCas9 in serum from the blood of 12 healthy adults and 22 cords blood. (Colque-Navarro et al. 2010; Dryla et al. 2005). These are consistent with a recent study detecting the presence of a humoral response against spCas9 and saCas9 with 10% and 2.5%, respectively, of pre-existing antibodies to these proteins in the serum of 200 healthy patients of American origin (Simhadri et al. 2018).

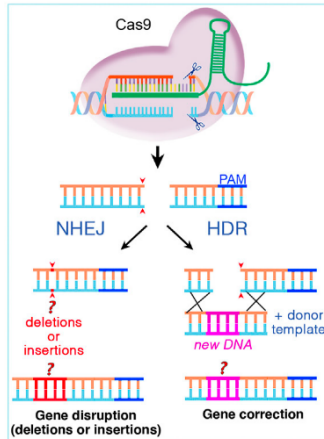
Furthermore, the presence of antibodies to Cas9 suggests the existence of memory T and B cells that elicit an adaptive immune response against Cas9 or cells with antigenic epitopes of the protein. This was confirmed by Charlesworth and colleagues, they detected the presence of T cells at a high frequency, within the population, directed against specific antigens of both Cas9 proteins (Charlesworth et al. 2019).

2. DNA- and RNA-based genome editing tools

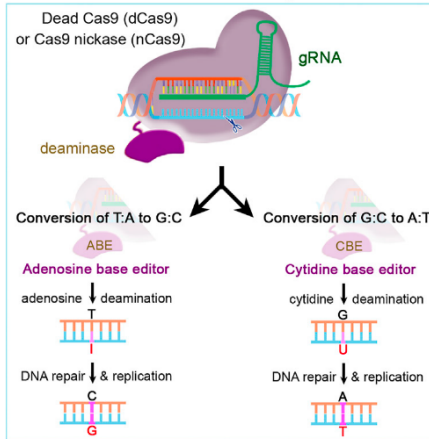
A new generation of gene editing systems recently emerged, broadening the scope of targetable sequences in the genome, and expanding their use beyond gene disruption and exon skipping (Anzalone et al. 2020). These new developments include the base and prime editors, which offer the potential for the effective and permanent correction of pathogenic mutations *in vivo*, in post-mitotic cells, without causing DSB and with the advantage of minimizing the formation of indels or off-target mutations (Levy et al. 2020). The DNA base editors developed by the group of David Liu induce the deamination of either cytosines or adenines, to allow for the repair of transition mutations (C-T or A-G changes) (Huang et al. 2021). Cytosine base editors (CBE) can convert C:G nucleotide pairs into A:T nucleotides (Komor et al. 2016) and adenine base editors (ABE) convert A:T into C:G nucleotides (Gaudelli et al. 2017). Prime editor (PE) has further expanded the CRISPR base-editing toolkit to all 12 possible transition and transversion mutations, and to small insertions or deletions (Anzalone et al. 2019) (**Figure 13A3**). This new process involves the use of an inactive Cas fused to a reverse transcriptase and an RNA called the pegRNA, which contains both the guide RNA and the correct template sequence (see Figure 5A3). Interestingly, prime editors are able to install point mutations at distances far (>30 bp) from the site of Cas9 nicking, which offers greater targeting flexibility than nuclease-mediated HDR with ssDNA donor templates, which typically are unable to introduce edits efficiently more than ~10 bp from the cut site (Anzalone et al. 2020). Finally, the recent progress in gene editing also involves new site-directed editing systems to edit RNA rather than DNA. This offers an improved safety profile, due to the transient and potentially reversible nature of edits made to RNA ((Fry et al. 2020; Rees and Liu 2018) (**Figure 13B**). Current progress in RNA editing technologies enabled the development of engineered enzymes capable of either adenosine-to-inosine (A-I, **Figure 13B2**) or cytosine-to-uracil (C-U) edits (Fry et al. 2020). Besides site-directed nucleobase editing, the use of inactive RNA enzyme editors tethered to various effector domains expand their applications to include regulation of RNA stability, splicing, intracellular localization, epitranscriptome modulation, translational activation/repression, RNA imaging, labeling of RNA-interacting proteins (**Figure 13B2**).

A Gene editing for CRISPR-DNA therapeutics (e.g. Cas9)

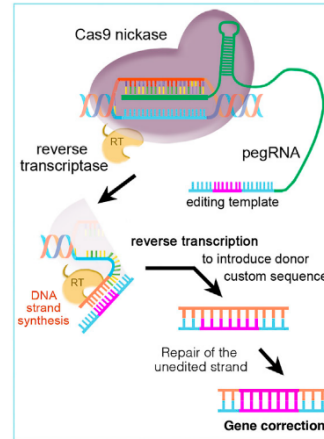
A1/ Cas9-mediated NHEJ and HDR



A2/ Base editing



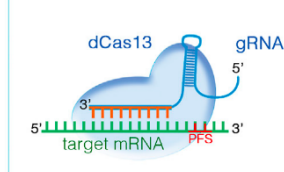
A3/ Prime editing



B Gene editing for CRISPR-RNA therapeutics (e.g. Cas13)

B1/ RNase defective Cas13 to:

- control splicing ?
- control translation
- RNA knock-down ?
- block ncRNA function ?



B2/ RNase defective Cas13-Fusion for: various applications, according to Cas-lethered effector domains:

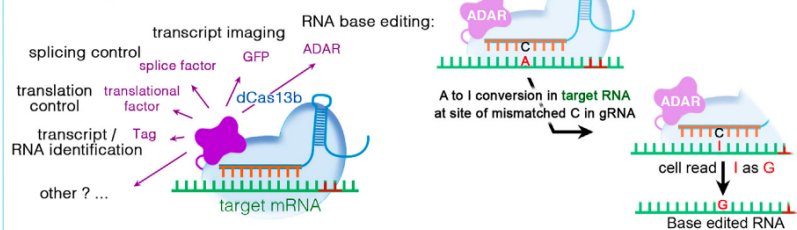


Figure 13 : DNA- and RNA-based genome editing to manipulate disease gene-of-interest. Different mechanisms occur, depending on the type of CRISPR/Cas nuclease and supplied editing tools.

(A1) The DNA CRISPR/Cas nucleases such as Cas9 or Cas12 can bind and cleave the genomic DNA. The double-stranded DNA breaks can then undergo repair by either the nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ is a random and highly error prone mechanism that incorporates insertion/deletion mutations, called indels, causing gene disruption (or silencing). In HDR, the DSB can be repaired by externally adding a donor DNA template that is homologous to the target sequence. The donor template is copied into the targeted site, resulting in a directed precise repair of the defective DNA sequence. (A2) Base editing relies on inactivated Cas nucleases (dCas) with preserved DNA targeting but without DNA cleavage, and could thus be used in non-dividing cells. The dCas nuclease (for instance Cas9 nickase, nCas9) is coupled to a deaminase, cytidine (CBE) or adenosine (ABE), which make it possible to edit specific nucleotide without DNA double break: C-to-T transitions by CBE editors or A-to-G transitions by ABE editors. (A3) Prime

editing is suitable to edit both point mutations and larger deletions or insertions. Here, dCas9 is fused with an engineered reverse transcriptase enzyme (RT), combined with a prime editing guide RNA (pegRNA) that serve to both position the enzyme at target site and provide the template sequence necessary to correct or replace the defective DNA region. (B) CRISPR Cas RNA systems, such as Cas13, can be used to manipulate cellular RNA both for basic research and therapeutics. While catalytically active Cas13 variants can cleave and disrupt the targeted RNA, the RNase-defective dCas13 (B1) and dCas13-effector fusion (B2) variants further expand possible RNA manipulations. These include regulation of RNA stability, splicing, intracellular localization, epitranscriptome modulation, translational activation/repression, RNA imaging, labeling of RNA-interacting proteins, or site-directed nucleobase editing (possible by ADAR effector domains), Abbreviations: ADAR, adenosine deaminase acting on RNA; PFS, protospacer flanking sequences; A, adenosine; T, thymidine; C, cytidine; G, guanosine; I, inosine; and U, uracile (Botto et al. 2021).

IV. Vectors for gene editing therapy in the retina

In vivo gene therapy delivery to the patient's targeted cell nucleus remains one of the main challenges in the field. For a gene replacement therapy, only one component needs to be transferred: the DNA of the functional protein. The CRISPR system and CRISPR derived gene editing tools (base and prime editors) are more complex and requires the transfer into the cell of multiple distinct entities: the Cas9 or editor system, one or two gRNA, and in some cases, a short DNA sequence (**Table 1**). In addition, gene editing tools questioned the necessity of a long-term expression of the delivered protein. Indeed, the persistence of the Cas9 protein can increase toxicity mediated by p53, potential off-target cuts and potential immune reactions against the edited protein and cells.

	SpCas9	SaCas9	Cytosine Base Editor (BE3)	Adenine Base Editor (ABE 7.10)	Prime Editor (PE3)
DNA (kb)	4.2	3.2	5.1	5.3	6.3
Amino acids	1367	1052	1699	1763	2098
Molecular weight (kg/mol)	160	130	200	201	240
Charge (at pH7)	23.2	34	15.8	20.3	32.2

Table 1: Characteristics of the main gene editing tools used.

Molecular weight and charge calculated from DNA sequence of each editing tool with PepCalc.com calculator.

1. Injection routes to the retina

The most appropriate method of administration depends on the area of the eye to be medicated. For retinal gene therapy in view of clinical application two local routes of delivery are preferred: intravitreal or subretinal (**Figure 14**). Today, subretinal injection is the route of injection allowing optimal targeting of photoreceptors and RPE, however injected region is limited. On the other hand, intravitreal injection is a less invasive injection route allowing pan-retinal delivery. However, this method leads to low transduction efficacy due dilution in the large vitreous volume and to low penetration across the inner limiting membrane. Indeed, limiting membranes and ECM can be critical factor for therapeutic efficacy.

Both subretinal dosing and intravitreal administration of gene therapy vectors in higher species induce mild and transient dose-dependent inflammatory responses. These responses seem to be greater in the vitreal compartment compared to subretinal space that offers better shielding of the vector from the immune system. Indeed, foreign protein and foreign DNA are known inducers of inflammation, which is also true in the immune-privileged ocular environment (Khabou et al. 2018).

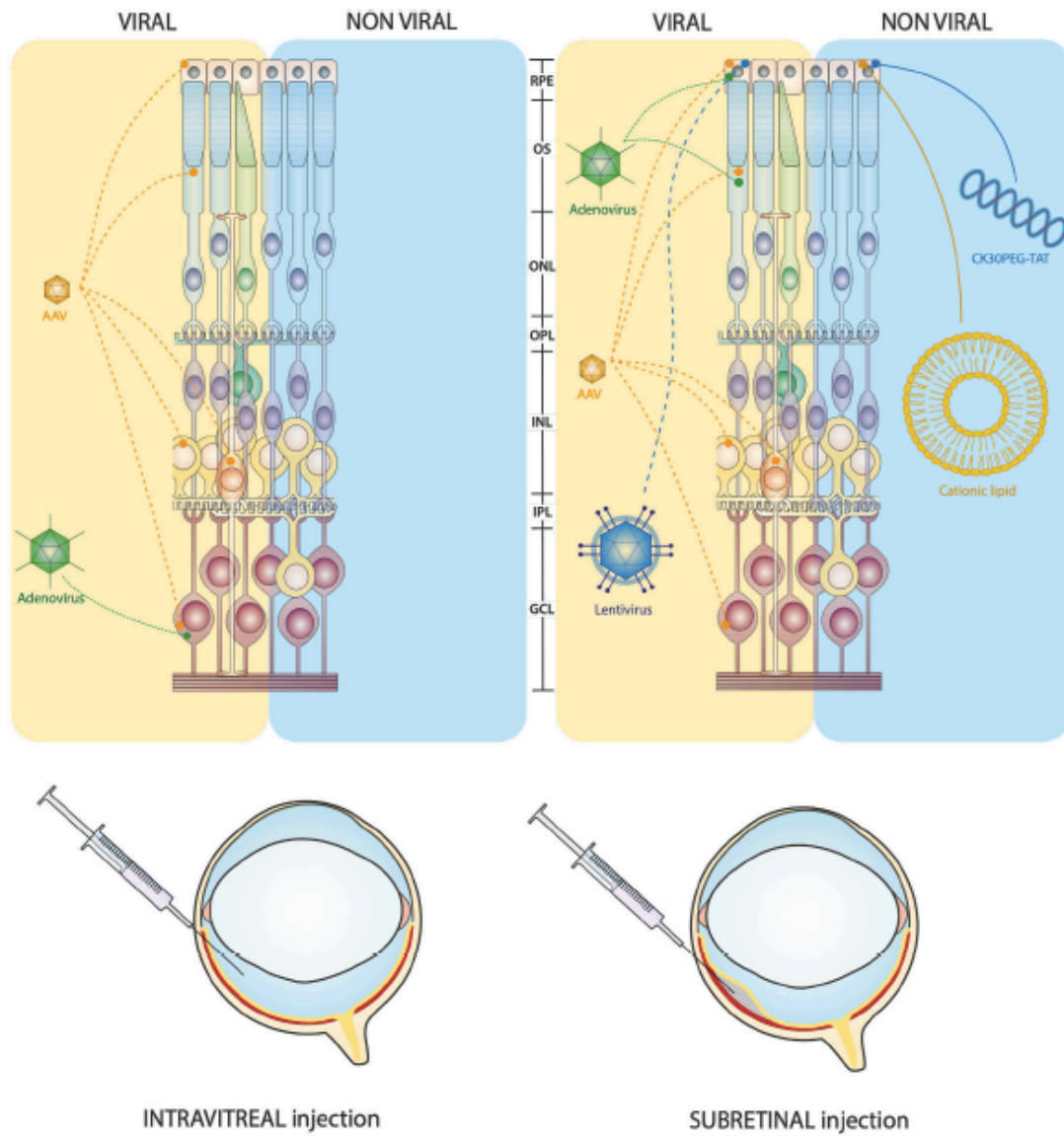


Figure 14 : Routes of administration and vectors used for each cell types.

A/Intravitreal and B/subretinal injection of viral (left) and non-viral (right) vectors into the retina From (Botto et al. 2022).

2. Vectors for gene therapy in the retina

Vectors are used in order to control spatial, temporal and quantitative distribution of a gene therapy towards a target. An ideal vector is efficient, stable, and safe and its production must be adapted to an industrial scale. Here, we will review main viral and non-viral vectors used *in vivo* for retinal gene editing delivery (**Figure 15**).

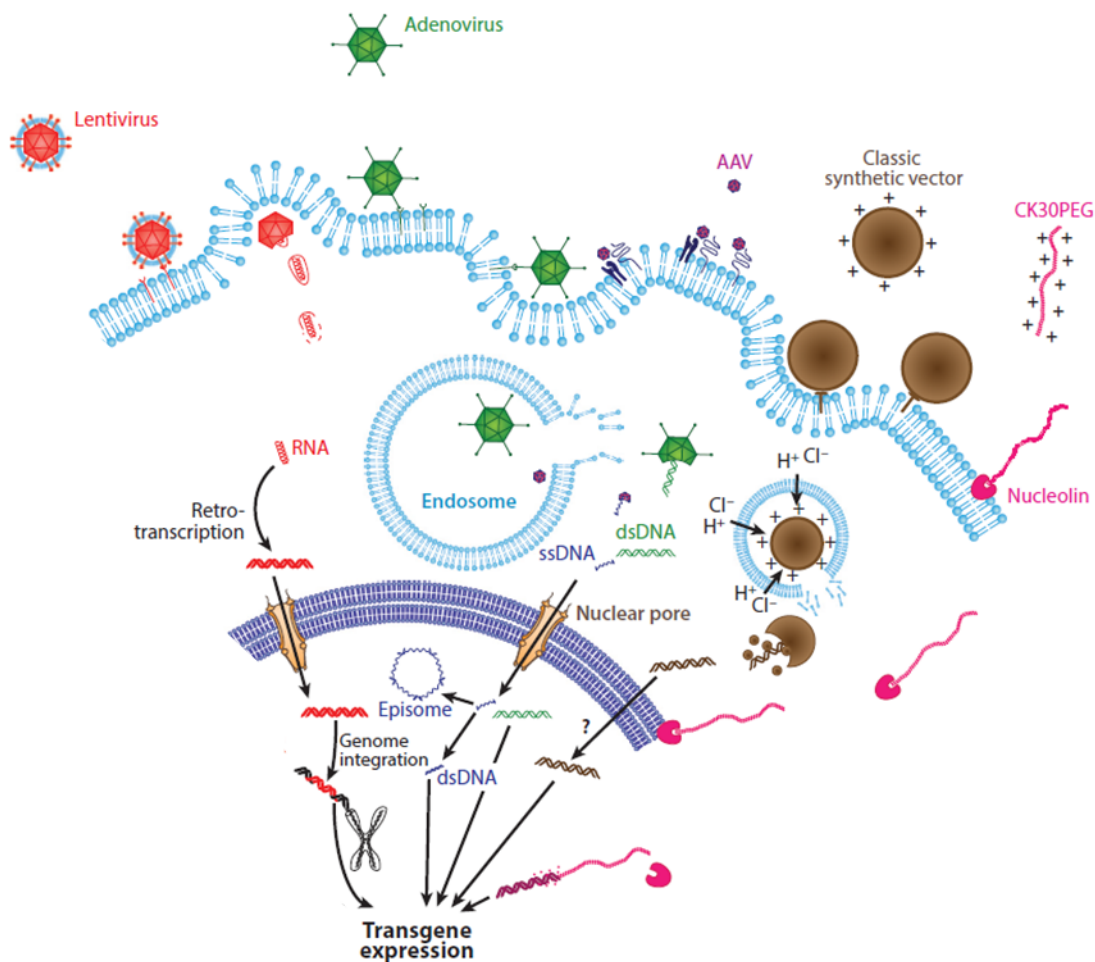


Figure 15 : Viral and non-viral vectors used in retinal gene therapy and their cell entry.

Adapted from (Planul and Dalkara 2017).

2.1 Adeno-associated virus

Adeno-associated virus (AAV) is a small (25 nm in diameter), non-enveloped vector containing a single-stranded linear DNA within its capsid. They have a carrying capacity of 4.7 kb and persists as an episomal DNA in post-mitotic cells.

The capsid of AAV is the most important parameter that determines which retinal cells will be transduced once the vector solution is delivered in proximity of the retina. It also plays a role in the immunogenicity of the vector. Depending on the administration route a capsid can reach multiple cell types, usually with a tendency to lead to most expression in cells it first comes into contact with. The specifics of which subpopulations of neurons are targeted also depends on the interaction of the AAV capsid with its primary and secondary cell surface receptors mediating its entry into the cells and trafficking into the nucleus. There are currently 12 AAV serotypes and over hundreds of naturally occurring or engineered variants that differ in their capsid composition (VP1, VP2 and VP3 proteins) (Schmidt et al. 2008). Based on the properties of their capsid, AAV serotypes reach different cell types depending on the species injected, the route of administration and its interaction with primary and secondary cell surface receptors.

Although RPE can be targeted by AAV, lentivirus or adenovirus vectors, only AAV vectors can pass efficiently the limiting membranes of the neurosensory retina. AAV2, 8 and 9 are the most frequently used natural serotypes to target photoreceptors after sub-retinal injection. Especially, AAV8 and 9 are able to target cone photoreceptors. To optimize their tropism and the transduction efficiency for a desired cell type, directed evolution has been used to obtain new AAV variants and allow targeting photoreceptors via less invasive methods such as intravitreal injections. Engineered variants AAV2-7m8 show capacity to transduce photoreceptor cells *in vivo* in mice when delivered via the vitreous alongside other variants obtained by directed evolution and rational design (S. L. Boye et al. 2020; Dalkara et al. 2013; Pavlou et al. 2021; Petrs-Silva et al. 2011; Reid et al. 2017) However, pan retinal gene delivery to the retina after intravitreal injections remain a problem in primate species with a thick inner limiting membrane (Byrne et al. 2020).

In addition, engineered AAVs allow targeting the hard-to-transduce inner nuclear layer neurons (bipolar, horizontal, and amacrine cells) (Cronin et al. 2014). Recently, AAVs have

also benefited from other design strategies such as in silico ancestral reconstruction (Carvalho et al. 2018) or with chemical modifications have been developed to improve gene delivery and could be used to modify AAV tropism (Mével et al. 2019; Thadani et al. 2020).

To obtain restricted gene expression of the transgene delivered by an AAV, specific promoters have been developed for each retinal cell type. Among them, *GRK1* (S. E. Boye et al. 2012) and *mCAR* (Busskamp et al. 2010) allow transgene product expression in all photoreceptors, whereas *PR1.7* (Ye et al. 2016) and *RHO* (Allocca et al. 2007) restrict expression to cones and rods, respectively. Thus, the serotype and promoter in front of expressed transgene are key parameters to target retinal cells in an efficient, safe and specific way. However, other parameters are involved in the cell specific targeting as the doses, the route of administration and state of the retina.

AAV has very low immunogenicity compared to other disease-causing viruses like Lv and Ad. Although immunogenicity of AAV is not a straightforward concept, because it depends on many factors such as transgene transported by AAV, promoter in front of expressed transgene, serotype of AAV, the dose of AAV or injection route, it has been used successfully for long term gene expression in several animal models and has shown efficacy results for a decade in the clinic (Hastie and Samulski 2015).

To sum up, AAV is without a question the vector of choice for retinal gene delivery with the majority of clinical trials using this vector for gene delivery. However, AAVs have a limited carrying capacity that prevent their applications to large genes or base and prime editors. Long term-expression of their transgene is no longer needed for gene editing strategies and could even increase potential immune reactions or toxicity.

2.2 Lentivirus

Lentiviruses (LV) are enveloped retroviruses of 80 to 100 nm in diameter containing a single-stranded RNA genome with 8 to 12 kB carrying capacity. They infect both dividing and non-dividing cells. After retrotranscription of their RNA in the cytoplasm, the viral DNA is inserted into the host genome at random sites. We can mention here that non-integrating lentiviruses (IDLV) have been obtained after mutation at the integrase level to avoid chromosomal integration.

Their relatively large capacity has allowed their use in multicistronic expression systems allowing delivery of multiple therapeutic proteins to the retina (Semple-Rowland and Berry 2014; Verrier et al. 2011; Williams et al. 2016) or for delivery of large transgenes to the RPE. Their diffusion across the inner and outer limiting membranes of the neural retina into the tight extracellular matrix is limited by particle size and composition. The development of the non-primate equine infectious anemia virus (EIAV) has raised hopes for overcoming this limitation forming the basis for the ongoing clinical trials for Stargardt disease (STGD) and USH type IB (USH1B) using this vector (Kachi et al. 2009).

2.3 Adenovirus

Adenoviruses (Ad) are icosahedral non-enveloped capsid containing a linear, double-stranded DNA genome with up to 37 kB carrying capacity. Vector genomes stay episomal and highly quality vector preps can be routinely manufactured but Ad's immunogenic nature have reduced their application in retinal gene therapy. A recent comprehensive study comparing the retinal transduction profiles of different naturally-occurring or mutant Ad serotypes shows that none of the Ad variants significantly outperforms Ad5 in adult murine PR transduction (Puppo et al. 2014). To date, no clinical trials based on Ad were undertaken for gene replacement, although two clinical trials have been performed using Ad5 intraocularly in order to treat age-related macular degeneration (Campochiaro et al. 2006) and retinoblastoma (Chávez-Barrios 2005). To sum up, adenoviruses can be a vector of interest for a gene therapy using a big transgene but they remain a second choice because of uncertainties about the expression level and longevity of the transgene particularly in larger animal models.

2.4 Non-viral vectors

Synthetic vectors are also often referred as nanoparticles (NPs) because of their sizes are in the nanometric range. These vectors are positively charged polymers, polysaccharides or lipids that interact with nucleic acids to compact and protect them into particles that can be internalized into cells. Synthetic vectors are easier to produce compared to biological agents reducing production costs compared to viruses (Trapani et al. 2014). Their derivation by changes in molecular structure can be easily implemented and they can carry up to 20 kb of DNA (Fink and Micol 2006), which makes them suitable for delivery of genes like ABCA4 or USH2A too large to be accommodated in AAV particles. Like AAVs and Ad, they do not provide integration into the genome, alleviating insertional mutagenesis. However, their cellular uptake and ability to transfer the DNA through the nuclear pores, is determined by their size and charge. Most studies of gene delivery to the retina using synthetic vectors to date used subretinal injections and only a few vectors seem to transduce efficiently the neural retina or the RPE without toxicity (Adijanto and Naash 2015). One of them is CK30PEG - a 30-mer cationic polylysine conjugated with 10-kDa polyethylene glycol (PEG), which produces rod-shaped compacted DNA NPs (Farjo et al. 2006; Fink and Micol 2006). These have been reported to deliver DNA to the RPE and photoreceptor cells (Han et al. 2012). Remarkably, their efficacy has been shown to last for up to 1 year in rodents albeit with lower gene expression levels than those obtained with AAVs. CK30PEG NPs are thought to be internalized by nucleolin, which transfers the compacted these particles seem largely responsible for their ability to get into the nuclei of retinal cells. As such, they were reported to lead to functional improvements in mouse models carrying mutations in photoreceptor-specific genes like the *Abca4*^{-/-} model of Stargardt disease (Han et al. 2012). Chitosan (a polysaccharide) NPs were shown to transduce photoreceptors and RPE after a subretinal injection and some retinal ganglion cells (RGCs) after intravitreal injection (Puras et al. 2013). Solid lipid nanoparticles (SLNs) are considered to be among the most effective lipid-based vectors. They consist of a solid lipid matrix surrounded by a layer of surfactants in an aqueous dispersion (Battaglia et al. 2016). Hyaluronic acid modified SLN transporting plasmids expressing GFP and RS1 were shown to transduce the RPE, the photoreceptors, and some RGCs and when carrying the human RS1 gene were able to improve the phenotype of

a mouse model of XLRS (Apaolaza et al. 2016). Nevertheless, the efficiency of these NPs seems lower than those obtained with viral vectors, particularly AAV. Another limitation of NPs is their formulation that can be difficult as some compounds are highly viscous preventing their wide distribution into the retina.

Current nanoparticle-based gene delivery in the eye has focused on using DNA as the therapeutic of choice. However, DNA delivery using nanoparticles is inherently inefficient due to the nuclear entry requirement in post-mitotic retinal cells.

There is an emerging need to develop non-viral vectors that can generate therapeutic levels of protein production especially for applications where transient presence of the protein is desirable as for gene editing applications.

The emergence of *in vitro* transcribed mRNA has expanded our ability to achieve high gene expression from mRNA by preventing immune response activation and stabilizing the mRNA thus expanding its half-life (Kowalski et al. 2019). mRNA mediated expression is thus an attractive strategy for delivery of endo/exonucleases for gene editing or activation of transcription factors for regenerative applications. *In vivo*, mRNA delivery to mouse photoreceptors has been described after the subretinal injection (Devoldere et al. 2019). A second study uses an ionizable lipid to deliver mRNA in the retina and shows high protein expression in the RPE (Patel et al. 2019; Ryals et al. 2020).

Although, the discovery of smaller Cas orthologues derived from *Staphylococcus aureus* (SaCas9) (Ran et al. 2015) and *Campylobacter jejuni* (CjCas9) (E. Kim et al. 2017a) allow its delivery with AAVs, the persistence of the Cas9 protein obtained via AAV mediated constitutive expression raises the possibility of permanent recombination in the genome, endogenous genetic disruption through long-term exposure to the encoded Cas9 protein, and potential immune responses (Charlesworth et al. 2019). Direct transfer of Cas9 mRNA or protein with its gRNA (RNP) can offer a transient presence of Cas9 which is degraded within three days (K. Kim et al. 2017c).

Indeed, it has already been shown that intracellular delivery of a Cas9 protein/gRNA complex using cationic lipids can target gene modification with high efficiency and greater specificity than administration of Cas9 DNA (Gao et al. 2018; Zuris et al. 2015). This approach has been shown to be effective in mouse inner ear cells (Zuris et al. 2015), in the retinal pigment

epithelium of the retina (K. Kim et al. 2017c) and recently in a mouse model of genetic deafness leading to phenotypic improvement (Gao, Tao et al. 2018). Kim et al., are the first to send Cas9 protein *in vivo* by a subretinal injection. They show a 22% indels of VEGFA in RPE cells leading to a therapeutic effect 7 days after the injection with an decreased CNV area (K. Kim et al. 2017c). There have not been any studies showing applicability in the neural retina thus far. In addition to delivery of nucleases, mRNA's ability to deliver large transgenes could be a modular alternative for base editors or prime editing tools that do not fit into a single AAV. Even if mRNA or protein do not face the same nuclear barrier as DNA delivery, many extracellular and intracellular barriers prevent efficient outcomes. The main challenges of the stability and immunogenicity of the mRNA are being sorted out by the *in vitro* transcription methods but variable in charge, size and shape than DNA or RNA. Therefore, different vectors are likely required for each editing system. Some commercial lipids have already been utilized (i.e.

Lipofectamine™ CRISPRMAX™ Cas9, ThermoFisher) but are for now only for *in vitro* use. The transport efficacy of synthetic vectors is still weak compared to AAV *in vivo* and more work is needed to improve the Cas9 mRNA or protein and sgRNA co-delivery to the retina for therapeutic benefit. These future improvements in mRNA and protein delivery systems also need to consider potential toxicity issues.

New category of non-viral vectors derived from virus: Nanoblades

Recently, a new vector for Cas9 protein transfer called Nanoblade was developed from murine leukemia virus (MLV), retrovirus family. These nanoblades do not contain viral DNA but only the Cas9 protein fused at the C-terminal to the GAG protein. Cas9 protein will also be able to complex to its gRNA within the nanoblade to form an RNP complex (Mangeot et al. 2019). This new vector has enabled the efficient transfer of these RNPs into RPE cells (Banskota et al. 2022). The correction matrix can also be complexed to the surface of the nanoblade and allows precise correction by homologous recombination. Although initially applied for *ex vivo* therapies, these new vectors open the way to in-depth modifications of viral vectors in order to perform transient delivery of Cas9 while allowing an efficient transfer.

To summarize, the need for an adequate delivery vector in order to apply safely and efficiently each gene editing tool in the neural-retina is still unmet. Delivery of protein or mRNA instead of DNA allows a transient delivery and bypass AAV limited carrying capacity. These two strategies have already been applied in the inner ear and the RPE. However, they have never been reported in the neural-retina tissue.

V. The challenges of gene editing in the retina

Over the last decade, improvements in the early identification of clinical clues suggestive of inherited retinal degeneration in children have decreased the time to diagnosis (de Bruijn et al. 2021). Early diagnosis is important, to ensure that visual rehabilitation is performed during the appropriate developmental window, both for further investigations and for the purposes of genetic diagnosis. Indeed, the timely recognition and optimal multidisciplinary management of these disorders can greatly improve the quality of life of the child, making it possible to use gene therapies, which are beginning to become available. Early genetic diagnosis also opens up the possibility of treatment before degeneration of the photoreceptors, the target harboring most of the genetic mutations leading to inherited retinal dystrophies. In this context, the CRISPR-Cas system and related gene-editing tools (base editing and prime editing) provide promising potential solutions, the application of which depends on the nature of the disease and the causal mutation. In the ClinVar database (ncbi.nlm.nih.gov/clinvar), ~84% variants associated with inherited retinal dystrophies are substitutions. Among them, ~34% and ~28% are respectively A-G or C-T transitions and can be repaired by cytosine and adenine base editors. In addition to single-nucleotide mutations, prime editing extends the capacity of gene editing repair to small deletions or insertions (~16% of all cases of IRD) and transversions (purine-to-pyrimidine changes, ~22% of cases). Thus, ~ 100% of pathogenic variants associated with IRD could be repaired by gene editing tools.

Many attempts have been made in recent years to demonstrate the efficacy of gene-editing tools for correcting human mutations, in cultured cell lines, and therapeutically relevant iPSCs with blindness- and/or deafness-associated mutations (Sanjurjo-Soriano et al. 2020). We focus here on the principal promising data obtained *in vivo* for this persistent challenge in biomedical therapeutics research.

1. Gene inactivation or excision of sequences containing mutations

By introducing cuts, the CRISPR-Cas9 system generates indels via the non-homologous end-joining (NHEJ) pathway, thereby triggering the knockout or excision of sequences containing mutations (**Figure 13A1**). This has proved to be the most easily applied strategy for the delivery of *SaCas9* and its gRNAs with a single AAV vector to the retina *in vivo*. The most advanced applications to date have involved the use of this technique to correct recessive Leber congenital amaurosis type 10 (LCA10), which is caused by an intronic mutation of the *CEP290* gene. The intronic c.2991+1655A>G mutation, reported in 20% to 57% of LCA10 patients of European descent, generates a new splice donor site leading to the insertion of a cryptic exon (exon X) in *CEP290* mRNA (p.Cys998X), leading to a premature stop codon in 50% of *CEP290* transcripts (Perrault et al. 2007). A combination of two gRNAs and a *SaCas9* that is under the photoreceptor-specific promoter GRK1 is delivered subretinally with a single AAV5, for direct excision of the intronic mutation. This method was shown to be safe and feasible in non-human primates with a dose dependent response and an efficiency superior to 10% productive edits, minimum threshold determined as clinically efficacious (Maeder et al. 2019). This study leads to the first clinical trial based on gene editing in the eye ([NCT03872479](#)).

Gene inactivation by a *Cas9/gRNA* DSB approach is also suitable for the treatment of disease due to dominant genetic defects, provided that the mutated allele is targeted specifically. Two strategies for the successful application of this gene inactivation *in vivo* in the retina have been developed, the first is based on an allele-specific PAM sequence present only in the mutated allele (Giannelli et al. 2018; Li et al. 2018), and the second is based on a mutation-specific gRNA (Bakondi et al. 2016). Representing 25% of dominant retinitis pigmentosa and 15% of all the retinal degenerations, Rhodopsin gene is among most frequently studied genes. The rod photoreceptor protein encoded by *RHO* gene binds retinal to initiate the phototransduction cascade in rod photoreceptors. Among all the mutations in *RHO* gene, P23H mutation is the most common one and cause rhodopsin protein misfolding and aggregation leading to a dominant-negative effect in rod photoreceptors. Two studies took the advantage of the engineered variant Cas9-VQR or its improved version Cas9-VRQR

that recognize the novel PAM NGA to precisely target the P23H mutated allele in knock-in P23H mice. Giannelli et al delivered by an intravitreal injection two AAV-PHP.B encoded, respectively, SaCas9-VQR under rod promotor and sgRNA with GFP reporter. They demonstrated a selective inactivation of P23H allele and an efficiency up to 27% indels among the highly GFP positive sorted cells. (Giannelli et al. 2018; Li et al. 2018) In the second study, Li et al found higher editing efficiency with the improved version Cas9-VRQR compared to Cas9-VQR. The majority of edited cell results in the knockout of P23H mutated allele and therefore the decrease of its transcripts (Giannelli et al. 2018; Li et al. 2018). To ensure an allele-specific inactivation, Bakondi et al based their strategy by two pair bases difference between the mutated and WT allele, one inside the PAM and one at position 10 in the 20 pair bases gRNA. This system allow the discrimination of the two alleles in a transgenic S344ter rat, a model of severe dominant retinitis pigmentosa characterized by rapid and progressive loss of outer nuclear layer from P11 to P28 (Bakondi et al. 2016). These three studies showed the potential of specific allele disruption to preserved the rods cells for dominant retinal diseases. However, these mutation-dependent strategies render treatments expensive and are beneficial to only a small number of patients. Moreover, it is not possible to compensate for haploinsufficiency due to inactivation of the mutant allele.

In this context, silencing and replacement strategies have been developed for dominant disease. The approach used involves the non-specific disruption of the two alleles, followed by their replacement via an AAV-based method. Latella et al. reported the inactivation of the *RHO* gene in neonatal P23H *RHO* transgenic mice after subretinal injection followed by electroporation of three plasmids encoding Cas9 DNA, two sgRNAs flanking exon1 and a GFP reporter. Among the GFP positive cells, a significant decrease in *RHO* protein levels was observed (Latella et al. 2016). In addition to the silencing of mouse *RHO* endogenous gene by Cas9 and two sgRNAs, Tsai et al. used a dual AAV approach to deliver a human *RHO* cDNA in postnatal P23H and D190N *RHO* knock-in mutant mice. Interestingly, they split the Cas9 and the sgRNAs into two AAVs and observed a correlation between the decrease of *mRHO* and the increase of *hRHO* levels. After three months, a phenotypic improvement was observed in the two models suggesting the possibility to apply this strategy for mutation-independent therapy. In a third study, McCullough et al. provided proof-of-concept for the gene disruption part of the strategy in non-human primate in the context of the autosomal dominant cone-rod dystrophy (CORD6). CORD6 is mainly caused by

mutations in *GUCY2D* gene coding for the retinal guanylate cyclase 1 (retGC1) (McCullough et al. 2019). Dual AAVs were used to encode *saCas9* under photoreceptor-specific promoter GIRK1 and a gRNA targeting *GUCY2D* gene associated with GFP reporter. Among sorted GFP positive photoreceptors, up to 13% editing efficiency (indels and AAV vector insertions) were observed, leading to 80% of retGC1 disruption. However, the second part of the strategy, involving the simultaneous replacement of the endogenous gene by a healthy gene, has yet to be reported and remains challenging (McCullough et al. 2019).

2. Precise correction

Precise correction has clear advantages over silence-and-replace strategies and would be applicable to diseases due to both dominant and recessive defects of large genes.

Precise correction via HDR pathway has been tested in a retinal degeneration 10 (Rd10) mouse model of autosomal recessive retinitis pigmentosa. In this model, a loss of function missense point mutations in *Pde6b* gene prevent the hydrolyse of cyclic guanosine monophosphate (cGMP) resulting in photoreceptor death due to cGMP toxic accumulation. By subretinal injection followed by electroporation, plasmids encodings GFP reporter, Cas9, gRNA and a single strand oligonucleotide (ssODN) were injected at day P3 and P8 in mouse pups during the pic and the end of the progenitor proliferation period respectively. Three days after injection, modest editing efficiency (~0.164%) was found in the whole retina, however optomotor test done at P90 showed the preservation of visual acuity in treated mice compared to non-injected mice (Vagni et al. 2019). An independent study reported similar results in neonatal rd1 mouse model carrying nonsense mutation in *Pde6b* gene by using a Cas9/RecA system that boost HDR pathway (Cai et al. 2019). These two studies show the potential of HDR repair pathway to edit precisely dividing photoreceptor progenitors. However, the NHEJ repair pathway predominates in post-mitotic retinal cells, and repair via the HDR pathway would therefore be unlikely to lead to a satisfactory correction rate *in vivo*.

The first strategy that could be used to overcome this limitation is the homology-independent targeted integration (HITI) strategy, which bypasses the HDR pathway and uses the predominant NHEJ pathway to accurately integrate sequences of interest at a given site

(Suzuki and Izpisua Belmonte 2018). In this approach, the Cas9/gRNA complex targets both the genome sequence and two short sequences inserted on either side of the donor template. Suzuki et al. provided proof-of-concept *in vivo*, in a rat model of retinitis pigmentosa. They used a dual AAV approach for the successful correction of a homozygous mutation of the *Mertk* gene in RP, leading to partial rescue of visual function (Suzuki et al. 2016). However, a mosaic of editing products can be generated by HITI strategy, as indels are nevertheless created by the NHEJ repair pathway.

Two studies have already reported the use of base-editing strategies on the retina in mice (Levy et al. 2020; Suh et al. 2021). In the first study, Levy et al. reported the use of a split-intein base editor-dual AAV strategy in mice expressing tdTomato only in rod photoreceptor cells (Levy et al. 2020). Robust base editing was reported in transduced rod photoreceptors suggesting that this delivery system could achieved therapeutically editing efficiencies *in vivo*. In the second study, a lentivirus expressing the ABE protein and its gRNA was delivered to the RPE of LCA mice model by subretinal injection, leading to ~16% of correction of a *de novo* nonsense mutation in the *RPE65* gene and the restoration of Rpe65 expression (Suh et al. 2021). To assess the rescue of visual function, retinal cell activity was measures using scotopic electroretinography (ERG), the recovery of 44% and 65% of a- and b- wave amplitudes were observed compared to WT control responses.

3. Gene editing to trigger pathway-induced cell reprogramming

It can be costly to correct each pathogenic variant individually. CRISPR-based gene-editing tools can be used for other purposes in mammalian cells, including the activation or repression of genes of interest, and the epigenetic reprogramming of cellular identities. Two groups have investigated the feasibility of a broadly applicable treatment for rod-cone dystrophies that is independent of the causal gene. This approach is based on repression of the *Nrl* gene, which can lead to the *in situ* reprogramming of rods cells into cone-like cells resistant to RP-specific mutations (Yu et al. 2017) (Moreno et al. 2018). Interestingly, Moreno et al delivered the CRISPRi system composed of a nuclease-deficient Cas9 (dCas9) and a gene repressor protein named KRAB by a dual AAV approach resulting in a similar effect than a knock-out strategy. A other gene-independent neuroprotective strategy was the first to target retinal ganglion cells to knock-down pro-degenerative gene (Wang et al.

2020). Genome editing is also a potentially powerful tool for the treatment of non-genetic degenerative diseases. Two studies reported an *in vivo* genome-editing approach for treating age-related macular degeneration (AMD) (A. B. Holmgaard et al. 2021; K. Kim et al. 2017b). Using cationic lipids vectors, they showed that Cas9/gRNA approaches based on disruption of the *VEGFa* gene can have therapeutic effects *in vivo* in a mouse model of AMD (K. Kim et al. 2017c). The development of gene-independent strategies based on CRISPR-Cas9 tools will make it possible to treat multiple inherited retinal dystrophies at early stages of the disease.

Aims

Genome editing mediated by CRISPR-Cas has shown promise for the treatment of retinal dystrophies (RD). Indeed, the broad range of gene editing tools being developed offers new therapeutic outcomes to treat all types of RD if suitable vectors are developed. Currently, adeno-associated viruses are the most widely used vector for retinal gene therapies but several limitations such as packaging capacity and long-term expression of Cas raise questions about their clinical applications for gene editing strategy. Transient delivery of gene editing tools could provide a safer alternative for delivering gene editing reagents to the retina. Successful delivery of Cas9 protein and its gRNA as ribonucleoprotein (RNP) complexes has been reported in the retinal pigment epithelium (RPE) and in the inner ear cells *in vivo* in mice. However, the feasibility of transient delivery of gene editing tools into the photoreceptors, the main target cells to treat early stage RD, have not been reported thus far.

The major aim of my PhD project was to investigate the feasibility of direct delivery of either Cas9 mRNA or Cas9 RNP to achieve gene editing in the photoreceptors and compare it to edition rates already achievable in the RPE cells. In the first part we focus on the evaluation of different non-viral vectors to complex Cas9 RNP and deliver it to photoreceptors and RPE in different models. Our overall goal was to correlate characteristics and efficiency of each RNP complex regarding its ability to edit retinal tissues. Same objectives were set for Cas9 mRNA delivery as part of the second part presented in chapter 2.

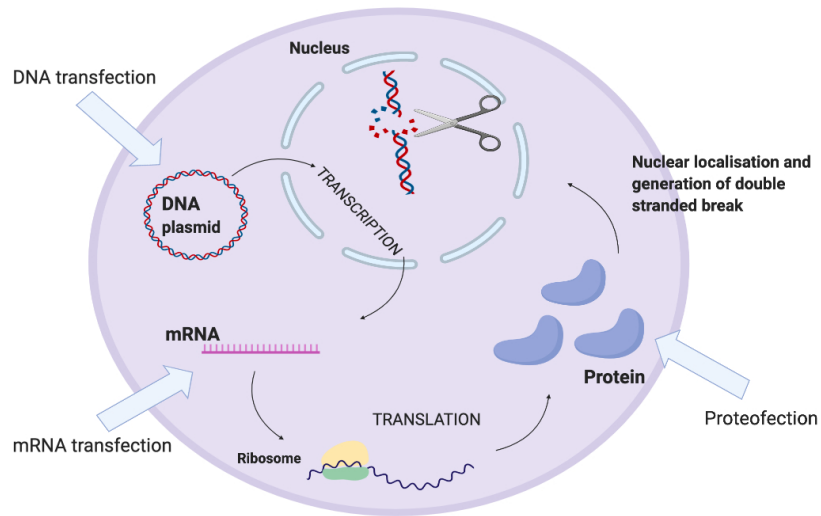


Figure 16 : Delivery of gene editing system in the cells as DNA, RNA or protein (Figure courtesy of Hanna Eriksson).

Our long-term goal with this approach is to apply these editing methods to Usher Syndrome type 1 B (USH1B) where an early intervention to edit causal mutations in photoreceptors is highly promising. Transient Cas delivery system developed here will be first applied to the retina and then to the inner ear of mouse and retinal organoid models of USH1B in the continuation of this work.

Results

Chapter 1: Cas Protein delivery in the retina

Manuscript in preparation:

Direct Cas9 protein mediated gene editing in the retina

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Abstract

Genome editing mediated by CRISPR-Cas has shown promise for the treatment of retinal dystrophies. Currently, adeno-associated viruses are the most widely used vectors for retinal gene therapies but their small packaging capacity and permanent transgene expression makes them suboptimal for CRISPR-Cas delivery. Transient delivery of gene editing reagents to the retina is preferable for therapeutic applications. Cas9 protein and its gRNA form ribonucleoprotein (RNP) complexes that can be delivered using cationic lipids to the retinal pigment epithelium (RPE) and into the inner ear cells *in vivo*. We investigated direct RNP delivery into photoreceptors as these are the target cells for most prevalent inherited retinal degenerations. Subretinal injection of Cas9 RNP generated indels in the neural retina in a dose dependent manner. Targeting *VEGFa* gene, the indels reached 0.4% in the whole neural retina compared 1.4% in the RPE. To improve delivery, we investigated different categories

of non-viral vectors such as cationic lipids and cell penetrating peptides (CPP). These synthetic carriers successfully complexed Cas RNP and increased its delivery to the RPE but not into the neural retina. RNP complexes showed accumulation above the tightly stacked outer segments pointing to physical barriers to neural retina infiltration. Delivery in mice lacking outer segments did not improve indel frequency suggesting other barriers such as the accessibility of the target gene and DNA repair mechanisms in degenerating photoreceptors are at play. Indeed, changing the target locus from *VEGFA* to *SAG*, a gene highly expressed in photoreceptors, improved significantly indel frequencies. Our results highlight the need to develop new carrier compounds and use suitable model systems for validation of gene specific interventions using CRISPR Cas.

Introduction

Gene replacement therapy has been successful for the treatment of inherited retinal degenerations (IRD) but it can only be used to treat monogenic recessive diseases caused by genes small enough to be delivered via AAVs. Allele-specific genomic ablation or gene correction using gene editing promise better therapeutic outcomes in diseases caused by dominant mutations or by mutations on large genes, which altogether affect a larger group of patients. To date, the most advanced gene editing application in the eye consists of the excision of a cryptic splicing site in the CEP290 gene to improve vision in patients suffering from Leber congenital amaurosis type 10 (LCA10). This strategy based on subretinal delivery of SaCas9 and two gRNAs with a single AAV vector has been successful in non-human primates (Maeder et al. 2019) and is the first clinical trial of gene editing in the eye (NCT03872479).

Unfortunately, viral mediated delivery of CRISPR-Cas-based editing reagents raises the possibility of permanent recombination in the genome (Hanlon et al. 2019), endogenous gene disruption through long-term exposure to the encoded Cas9 as well as potential immune reactions to these endonucleases with bacterial origin (Charlesworth et al. 2018). In addition, 4.7 kB carrying capacity of AAV limits the use of new gene editing tools such as base and prime editors that can be used to treat up to 99.9% of IRD (Anzalone et al. 2019; Gaudelli et al. 2017; Komor et al. 2016).

A non-viral delivery strategy using Cas9 protein and its sgRNA as ribonucleoprotein (Cas9 RNP) was first reported in 2015 (Zuris et al. 2015). Cas9 RNP complexes were delivered using cationic lipids to inner ear cells of mice *in vivo* (Zuris et al. 2015) and later in a mouse model of genetic deafness, in which it led to phenotypic improvement (Gao et al. 2018). This concept was first applied to the retina by Kim et al., 2017, who showed that cationic non-viral vectors were able to deliver Cas9 protein to the retinal pigment epithelium (RPE), resulting in indels in 22% of the transfected cells (K. Kim et al. 2017b). A second study confirmed the capacity of lipid vectors to deliver Cas9 protein to the RPE, to disrupt the *VEGFA* gene. A more modest indel rate of 6% was achieved in the *VEGFA* locus in isolated EGFP positive RPE cells (A. B. Holmgaard et al. 2021). However, signs of toxicity at high Cas9/gRNA concentrations were reported (A. B. Holmgaard et al. 2021). A more recent study

reported efficient genome editing in the RPE by conjugating a new carrier with all-trans retinoic acid which are selectively transported to RPE by the inter-photoreceptor retinoid-binding protein (Chen et al. 2019). Although these first attempts at transient gene editing in the RPE are promising, so far there are no reported studies of RNP mediated gene editing in cells of the neural retina. As photoreceptor cells bear the majority of mutations leading to inherited retinal degenerations where gene editing will find applications in the years to come, we investigated RNP delivery to the neural retina using this direct delivery strategy.

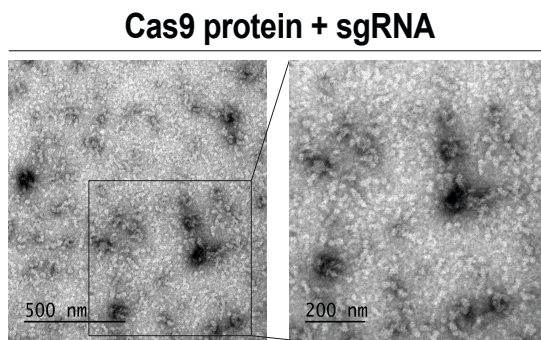
Here, we investigate transient delivery of Cas9 protein as RNP in the whole neural retina in comparison to RPE cells. We tested different categories of non-viral vectors with different physico-chemical properties giving a broad range of size and charge. We compared their ability to complex and deliver RNP into photoreceptor cells and in relation to their ability to deliver the cargo to RPE and thereby identify target cell-specific barriers. These barriers include physical barriers such as outer segments and outer limiting membrane shielding the neural retina from the subretinal space as well as intracellular barriers such as the accessibility of the targeted gene within the genome. Our results show the importance of the size of RNP complexed with vectors in entering the neural retina and in limiting their toxicity. Direct RNP delivery to the neural retina gives the best indel frequency whereas RPE cell entry is aided by the use of cationic lipids. Moreover, through a series of experiments on other relevant models as human and macaque retinal explants we highlight same barriers specific to the neural-retina tissue. Altogether our work highlights the key parameters to consider in designing new classes of carrier compound to bring RNPs into the retina for therapeutic gene editing. Moreover, it highlights the cell and gene specific features of CRISPR Cas mediated gene editing that must be taken into account in the design and preclinical testing of CRIPR therapeutics.

Results

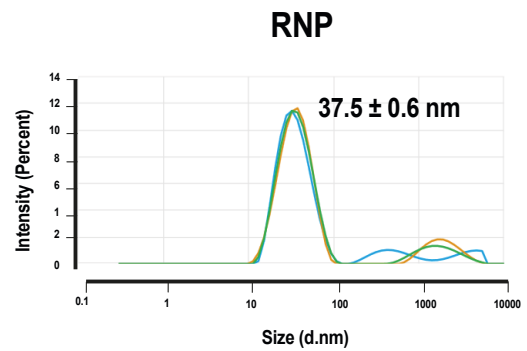
Ribonucleoprotein complexes (Cas9 protein and its sgRNA) induce indels in the neural retina and in the RPE.

As an alternative to gene delivery using AAVs, here we attempt to directly deliver Cas9 as a protein coupled with its gRNA. We used a Cas9 protein from *Streptococcus Pyogenes* (net charge +22) with two nuclear localization signals (NLS, net charge +5) and complexed it with an sgRNA targeting *VEGFa* gene (net charge -120) forming an RNP complex with a net charge -88. Cas9 proteins with two NLS tag gave rise to homogenous RNP complexes as observed in electronic microscopy and reported by dynamic light scattering (DLS) with a unique peak with a mean at 37.5 nm, coherent with previous reports (Nguyen et al. 2020) (Fig. 1 A and B). Next, we performed sub-retinal injection of sgRNA and Cas9 protein in adult wild-type mice. We analyzed indels induced *in vivo*, one week after injection of Cas9-2NLS protein with its sgRNA. The whole RPE and the whole neural retina were isolated without any cell selection and indels at the target site were quantified by NGS analysis (Fig. 1B). Direct delivery of Cas9-2NLS RNP generated indels in the RPE and in the neural retina in a dose dependent manner with the best efficacy at 30 uM (Fig. S1A). One week post-injection, 0.4% indels were generated in the whole neural retina compared to 1.4% in the RPE (Fig. 1C). The indels pattern we observed was a 1 base pair (bp) insertion (T), 3 bp away from the PAM sequence (Fig. S1B). Based on results from previous reports, we quantified the indels after a few weeks interval. Interestingly, percent indels doubled between one week and one month after injection reaching 1.1% in the RPE and 2.3% in the neural retina indicating different time scales for intracellular entry and subsequent indel generation (Fig. 1C).

A.



B.



C.

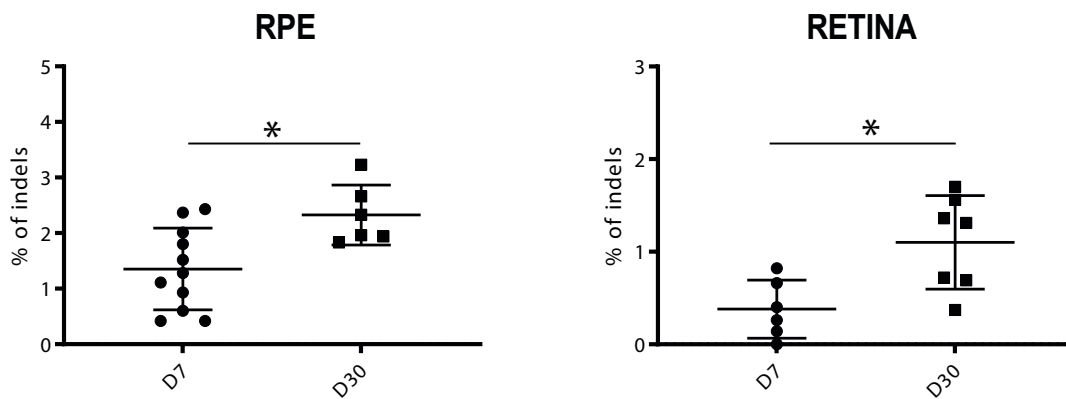


Figure 1 : Direct RNP delivery induces indels *in vivo* in the neural retina and in the RPE.

A/ Cas9-2NLS RNP (30uM, ratio 1:1) negatively stained with 2% uranyl acetate for 4 minutes and observed by electronic microscopy. Scale bar: 500nm (left) and 200nm (right).

B/ Size of Cas9-2NLS RNP (30 uM, ratio 1:1) determined by DLS. Three different measures (in blue, red and green) were performed at 5-minute intervals.

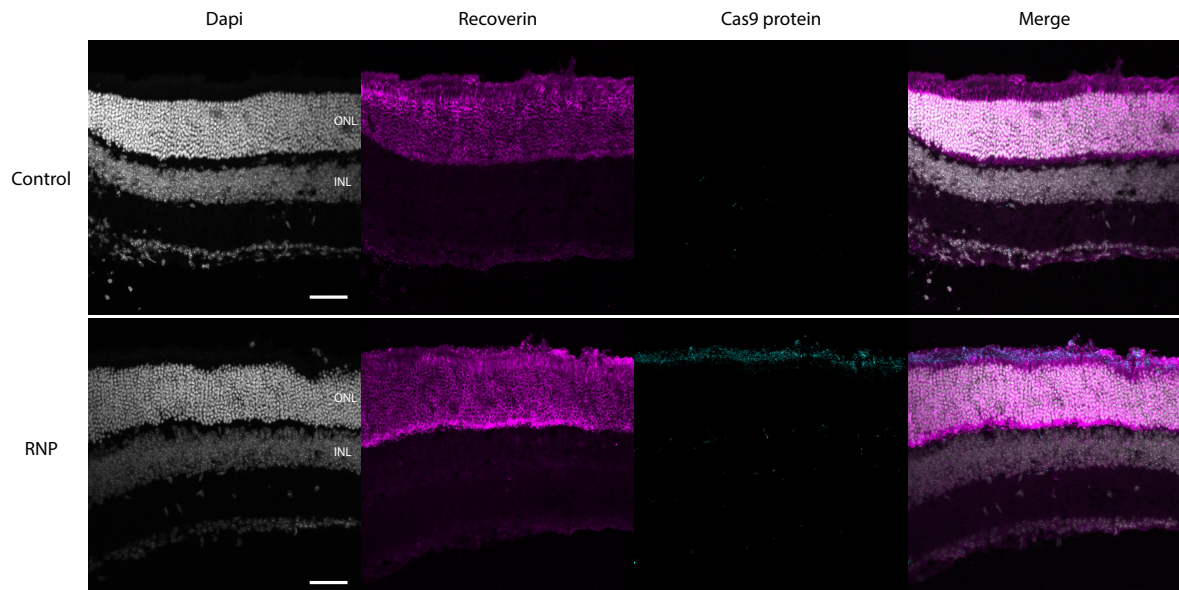
C/ Percentage of indels in the whole RPE and neural retina after sub-retinal injection of Cas RNPs (30uM, ratio 1:1). NGS analysis was performed 7 days after injection. Mean $\pm$ SD.

Student's t-test: * $p < 0.05$

Physical barriers of neural retina prevent Cas RNP delivery *in vivo*.

To document further the obtained result, we examined three days post-injection the distribution of the Cas9 complexes in the retina. Injections with Cas9 RNP complexes in adult wild-type mice showed that the majority of Cas9 protein was not able to infiltrate into the photoreceptor layer and reach the outer nuclear layer (ONL). By staining the cytoplasmic membrane of the photoreceptors (PRs), we observed that the majority of the Cas9 proteins are blocked into external segments of PRs and prevent its delivery into the ONL. Indeed, after a sub-retinal injection, Cas9 protein complexes encounter specific barriers of the neural retina as outer segments discs, external limiting membrane and tight ECM of the outer nuclear layer that likely prevent diffusion of nanoparticles. To understand if physicochemical barriers specific to photoreceptors prevent the delivery, Cas RNP was delivered in a retinal degeneration rd10 mice model at a stage where degeneration of the outer segment is advance (Rösch et al. 2014). Three days post-injection, no Cas9 proteins were observed by immunostaining (Fig. 2A). However, no indel increase was seen compared to wild-type mice suggesting other post-entry barriers are involved in this mouse model, precluding efficient editing with Cas9 (Fig. 2B).

A.



B.

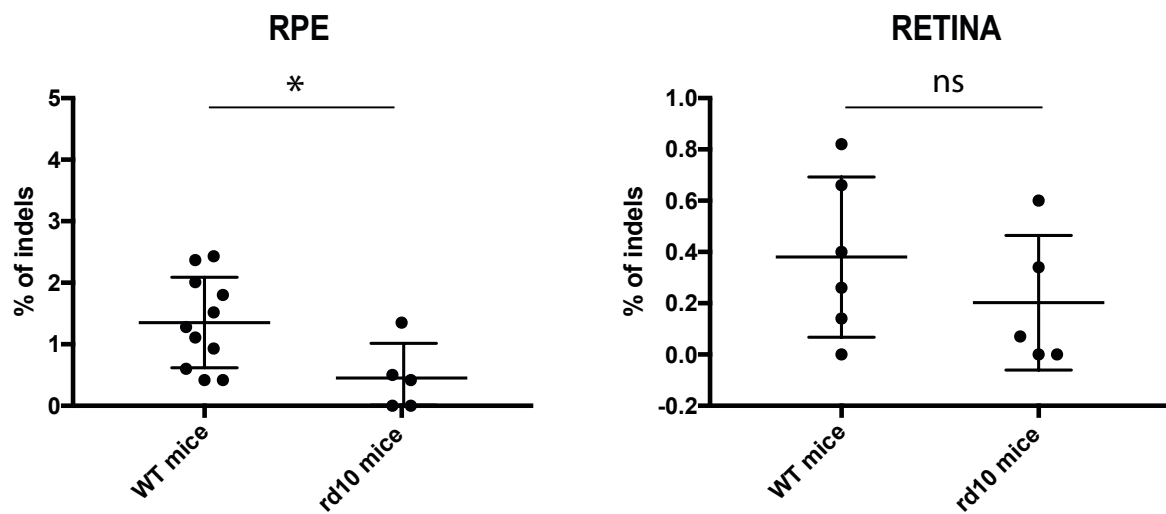


Figure 2 : Localization of Cas9 protein inside the neural retina and indels generated after RNP delivery in wild-type mice compared to rd10 mice.

A/ Localization of Cas9 protein inside the neural retina after 2uL subretinal injection of Cas9 RNP (30 uM, ratio 1:1) in C57Bl6J WT mice. Neural retina cross-sections were stained for nuclei (Dapi, gray) cytoplasmic membrane of PRs (recoverin, magenta), Cas9 protein (cyan). Each condition was done in duplicate. Scale bar: 25um. ONL: Outer nuclear layer, INL: Inner nuclear layer.

B/Percentage of indels induced in the whole RPE and the whole retina after 2uL sub-retinal injection of Cas RNP (30uM, ratio 1:1) in WT mice and rd10 mice injected at D39. NGS analysis was performed 7 days after injection. Mean $\pm$ SD. Student's t-test: $p < 0.05$.

Complexation of Cas9 RNP with lipid vectors

To improve editing efficiency and bypass physical barriers specific to the retina identified above, we investigated the potential of different categories of non-viral vectors to facilitate delivery into retinal cells. Indeed, RNP entry into the cells may not be favored due to the negative global charge of these complexes (Zuris et al. 2015). To enter cells by endocytosis or direct penetration through the cell membrane after interaction with proteoglycans, positive surface charges are preferred.

Based on previous studies in the inner ear and in the RPE, we investigated the capacity of the commercial synthetic carriers Lipofectamine 2000 or RNAiMAX to deliver Cas9 RNPs to neural retinal cells (K. Kim et al. 2017b; Zuris et al. 2015). We measured the size of RNP-lipofectamine 2000 complexes by DLS and observed polydisperse complexes from 24.6 ± 5 nm to large complexes 1910 ± 252 nm (Fig. 3B and Fig. S2). We performed sub-retinal injections of Cas RNP complexed with lipofectamine 2000 or RNAiMax in adult WT mice and quantified indels 7 days post injection. In the RPE, RNPs complexed with lipofectamine 2000 or RNAiMAX generated indels with a mean at 2.3% and 2.1 % respectively (Fig. 3C). Thus, the cationic carriers such as lipofectamine 2000 improve slightly Cas9 RNP delivery into the RPE. In the retina, we do not obtain improvement compared to RNP alone with this carrier with an indel rate remaining below 1% (at a mean of 0.97). However, we notice a large variability between samples with an isolated point around 2.6% (Fig. 3C). Taken together, these results indicate that Lipofectamine2000 and RNAiMAX improve Cas9 RNP delivery into the RPE but not into the neural retina.

We hypothesized that the large and heterogeneous size of the lipoplexes could be the reason for lack of improvement in delivery into the neural retina. In order to decrease the size of our complexes and promote diffusion across the tight intracellular matrix of photoreceptors and intracellular entry of the RNPs, we tested a lipo-peptide recently reported to efficiently complex RNPs and deliver them to several cell types *in vitro* (Jain et al. 2019). This lipo-peptide is composed of a lipid tail linked to a tandem peptide carrying a net positive charge. As expected, the size and polydispersity of the complexes decreased by the implementation of this lipopeptide. Complexes of 208 ± 47.1 nm were obtained (Additional

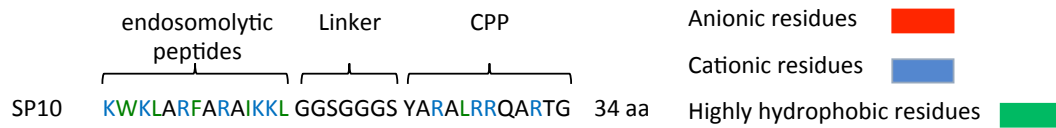
results) and injected sub-retinally in WT mice. With this approach indel rates were lower compared to both RNP alone and lipofectamine-RNP in the neural retina (Additional results).

Cas9 RNP delivery with shuttle peptides

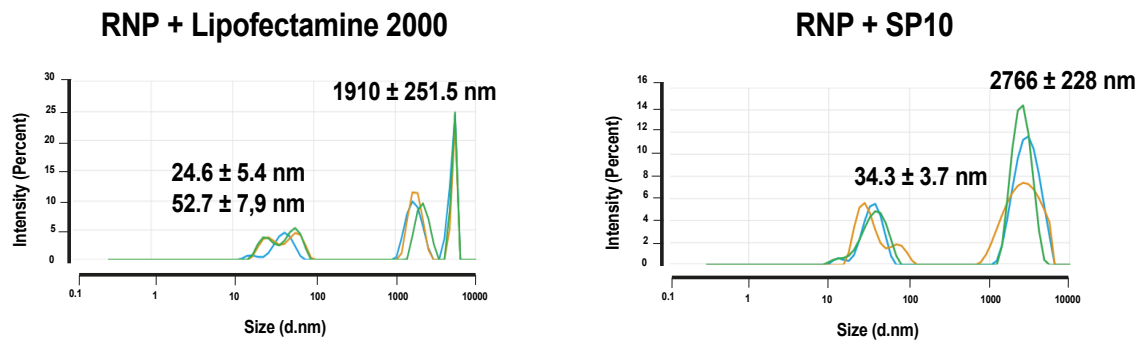
Based on the data presented above, use of lipopeptides helped decrease size and polydispersity of the complexes compared to lipid alone but this did not allow more efficient RNP delivery into the neural retina. Lipids may not be suitable for delivery into the photoreceptors across the physical barriers. Indeed, even lipid-enveloped viruses such as lentivirus have a difficult time transducing the neural retina (Grüter et al. 2005). We thus investigated the possibility to increase the delivery by switching to a peptide-based system. A more recent study showed the capacity of a shuttle peptide (named SP10) to complex and deliver Cas9 RNP into epithelial cells *in vivo* (Krishnamurthy et al. 2019). This polypeptide is composed of a cell-penetrating peptide (CPP) linked to an endosomolytic peptide that transiently destabilizes endosomes (Fig. 3A; net positive charge + 10). Mix of SP10 peptides and Cas9 RNPs formed heterogeneous complexes from 34.4 ± 3.7 nm to large complexes of 2766 ± 288 nm (Fig. 3B). To determine if this polypeptide SP10 can deliver Cas9 RNPs to the RPE, RNPs (ratio 1:1, 30 uM) were mixed with SP10 (250uM) and were injected into the subretinal space of WT mice. At 7 days post-injection, we observed indels at a frequency 0.76 to 2.81 % in the RPE and 0.4% in the whole retina (Fig. 3C).

Taken together, these results show that mix of Cas RNP with peptides seems not to lead to efficient complexation and thus efficient edition in the retina. Covalent bonds between Cas9 protein and peptides could facilitate the formation of small and homogenous complexes and aid delivery.

A.



B.



C.

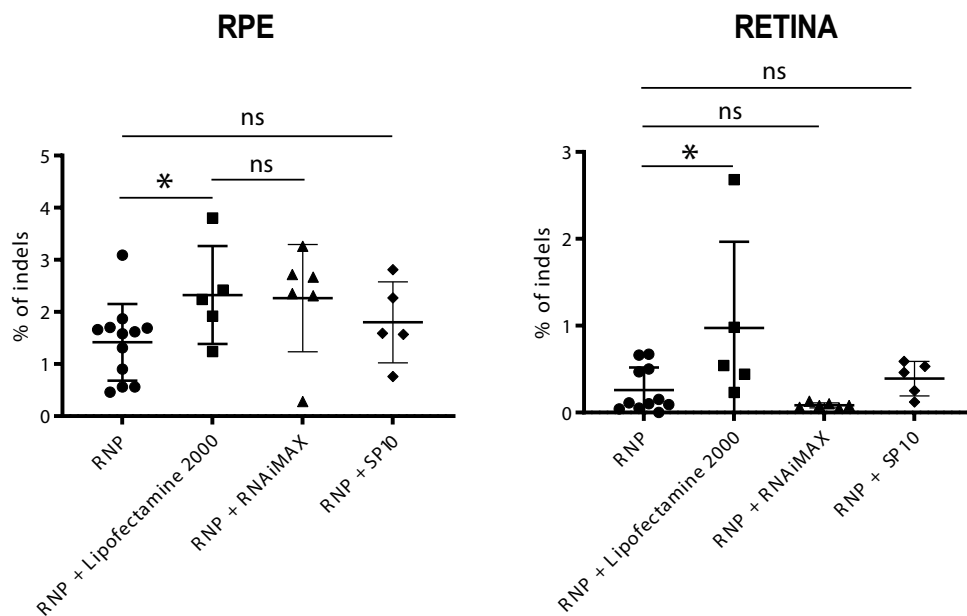


Figure 3: RNP delivery using shuttle peptides (SP10) in the retina.

A/ SP10 peptide sequence of 34 amino acids. Anionic amino acids are highlighted in red; cationic amino acids are in blue and highly hydrophobic amino acids are in green.

B/ Size of Cas9-2NLS RNP (30 uM, ratio 1:1) complexed with Lipofectamine 2000 or SP10 peptide determined by DLS. Three different measures (in blue, red and green) were performed at 5-minute intervals.

C/ Frequencies of indels induced in the whole RPE and retina after 2uL of sub-retinal injection of Cas9 RNP (30uM, ratio 1:1) complexed with non-viral vectors: Lipofectamine 2000 or RNAiMAX or SP10 (250uM) *in vivo in WT mice*. NGS analysis was performed 7 days after injection. Mean $\pm$ SD. Student's t-test: * $p < 0.05$, ns: non significant.

Delivery of Cas9 RNPs triggers some toxicity inside the retina.

Cas9 being a protein of bacterial origin, there are risks associated to injecting it at high levels into the retinal tissue. We thus investigated the potential toxicity due to RNP delivery inside the retina by *in vivo* retinal imaging and post-mortem histology on treated retinas. First, we performed optical coherence tomography (OCT) 1 month after Cas9 RNP sub-retinal injection. We measured some infiltrations in the vitreous and a non-significant decrease of the retinal thickness in eyes injected by Cas9 RNPs (Fig. 4A,B and Fig.S3). To investigate microglial activation, we performed Iba1 staining on whole-mount retinas one-month post injection. We observed more activated microglial cells in RNP injected retinas compared to buffer injected retinas (Fig 4C). Here, we highlight the necessity to investigate immune reactions against Cas RNPs in the retina to evaluate their full potential. Development of new and efficient carriers will allow to protect Cas protein and decrease the quantity of RNP necessary for a therapeutic effect thereby decreasing immune cell activation.

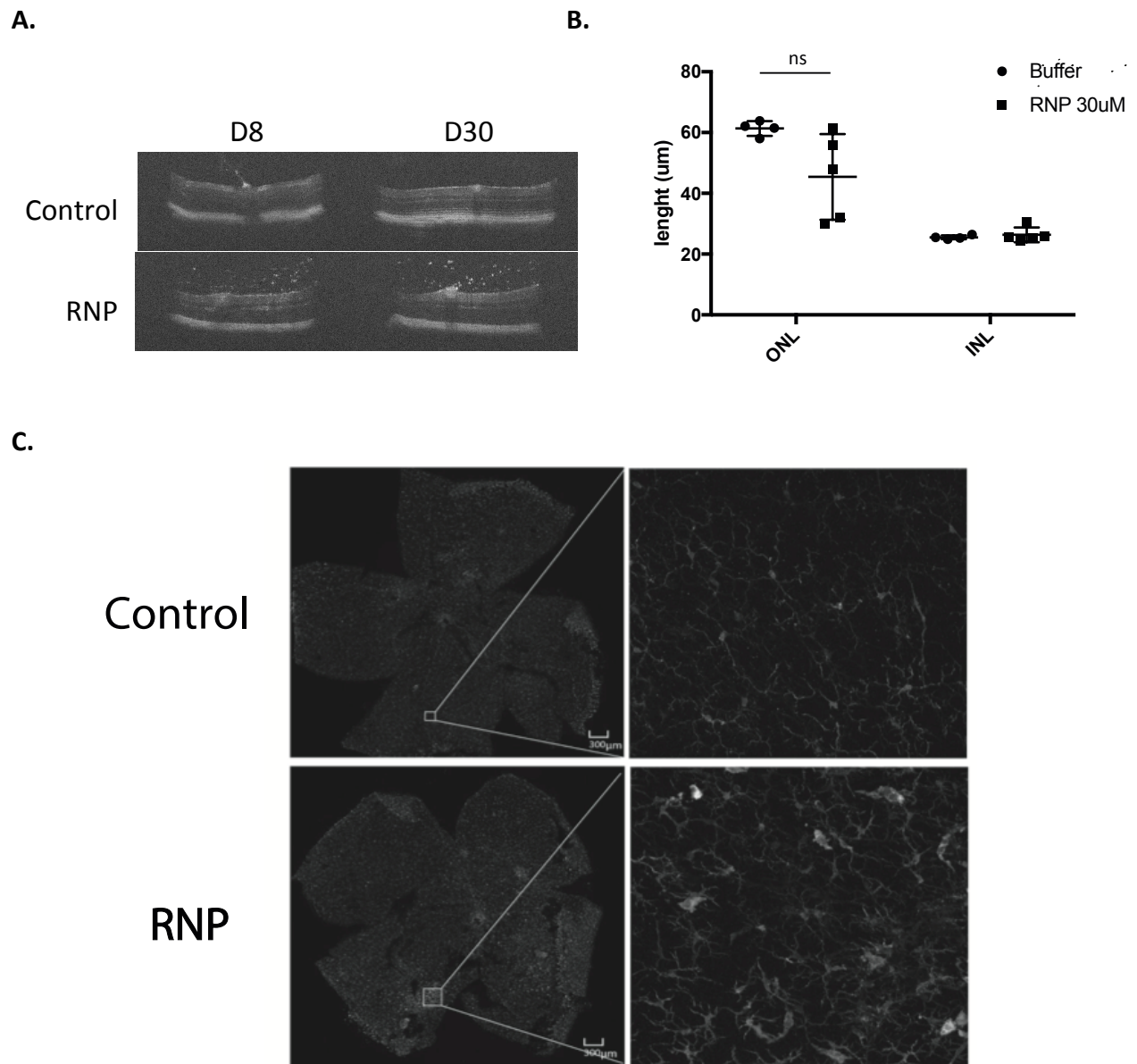


Figure 4: Toxicity induced by RNP delivery 1 month after sub-retinal injection.

A/ OCT images 1 month after injection of buffer solution or Cas9 RNP (30uM) in WT mice.

B/ Length of outer nuclear layer (ONL) and inner nuclear layer (INL) analyzed based on OCT images (central images) in eyes injected by Cas9 RNPs compared to control eyes. Mean $\pm$ SD. Student's t-test, ns: non-significant.

C/ Microglia cells activation after subretinal injection of Cas9 RNPs (30 uM, ratio 1:1) in adult WT mice. Flat mount retinas were stained for nuclei (Dapi, blue), microglia cells (green). Scale bar, 30um (left) and 75um (right).

Delivery of RNPs in macaque and human retinal explants.

As previously highlighted, it is important to test gene editing agents on relevant cell targets of relevant species in order to better predict potential therapeutic effect in clinical applications. *VEGFA* gRNA that we used for this study have the advantage of targeting a sequence common to human, macaque and mouse genome allowing comparisons of targeting efficacy across different species in fully differentiated photoreceptor cells (K. Kim et al. 2017b). Thus, we further tested Cas9 RNP delivery systems *ex vivo*, in human and non-human primate retinal explants. A 2 μ L drop was deposited above the photoreceptors of each explant and indel rate and localization of Cas9 were determined 3-7 days later. Cas9 proteins were found above the ONL in non-human primate retinal explants indicating that similar to mouse studies *in vivo*, Cas9 RNP is not able to go through the photoreceptor layer on its own or when complexed with lipofectamine 2000 (Fig. 5). However, in contrast to *in vivo* mouse studies, no indels were detected in non-human primate or human retinal explants (Table S1). No indels were found with Cas9 RNPs complexed with lipofectamine 2000 or SP10 (Table S1). To understand if this difference in efficacy was a result of going from an *in vivo* to an *ex vivo* explant system, we compared these results to results obtained under similar conditions in mouse retinal explants. We found an indel rate of 0.35% in mouse explants, similar to *in vivo* studies, in one of the three explants tested. This shows there is a greater variability in indel frequencies in explant conditions probably due to altered endocytosis and DNA repair mechanisms at play in explants compared to *in vivo* conditions. Detailed experimental conditions and NGS analysis done on retinal explants in this study are listed in Supplementary Table S1.

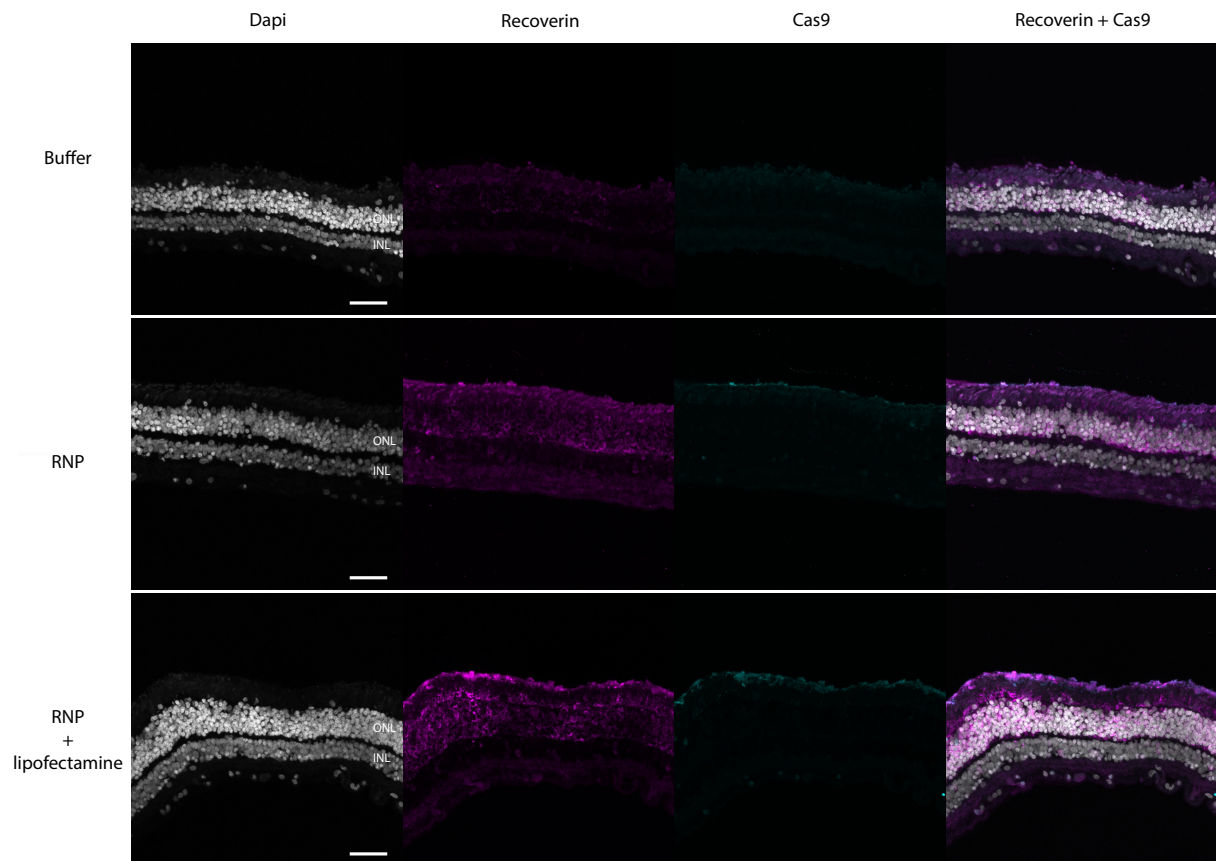


Figure 5: Cas9 RNP delivery in macaque retinal explants.

Localization of Cas9 proteins inside the retina by immunostaining. Retinal explants were incubated with Cas9 RNPs (30uM) alone or complexed with Lipofectamine 2000. After 3 days, explants were fixed. Cross-sections of retina were stained for nuclei (Dapi, blue), Recoverin (green) and Cas9 protein (Red). Scale bar, 25um. ONL: Outer nuclear layer, INL: Inner nuclear layer.

Cas9 RNPs induce a high percentage of indels in a gene highly expressed in photoreceptors.

To better understand the barriers in obtaining gene editing in photoreceptors, we decided to change our guides from *VEGFa* to guide RNAs targeting relevant genes highly expressed in the photoreceptors. Such highly expressed genes are also often involved in retinal dystrophies (Pulman et al., 2022). Several sgRNAs were designed and validated in a murine cone cell line by TIDE assay. The sgRNA 3 in exon 8 of the *SAG* gene induced indels with the highest frequencies compared to other sgRNAs (Fig. S5). Among the three highly expressed photoreceptor genes (*SAG*, *RHO* and *PDEb*) (Fig. S4), we selected *SAG* gene coding for S-arrestin protein associated with autosomal recessive stationary night blindness (Nishiguchi et al. 2019) and retinitis pigmentosa phenotype depending on the mutation (Sullivan et al. 2017). Mutations in *RHO* and *PDE* gene also lead to retinitis pigmentosa with rhodopsin mutations accounting for 30% of all retinitis pigmentosa disease but the editing efficiencies in these genes were lower *in vitro*.

We further tested the best sgRNA targeting *SAG* gene *in vivo* and compared it to previous sgRNA targeting *VEGFa* gene. After 7 days, we observed 7.6% indel rate in the neural retina compared to 0.5% with *VEGFa* sgRNA. This remarkable increase in indel rate is corroborated with a high decrease in *SAG* mRNA in the neural retina compared to non-injected eyes (Fig. 6). Taken together, these results show the potential of Cas9 RNPs to efficiently edit neural retina when coupled to guides that have a high activity in an active locus.

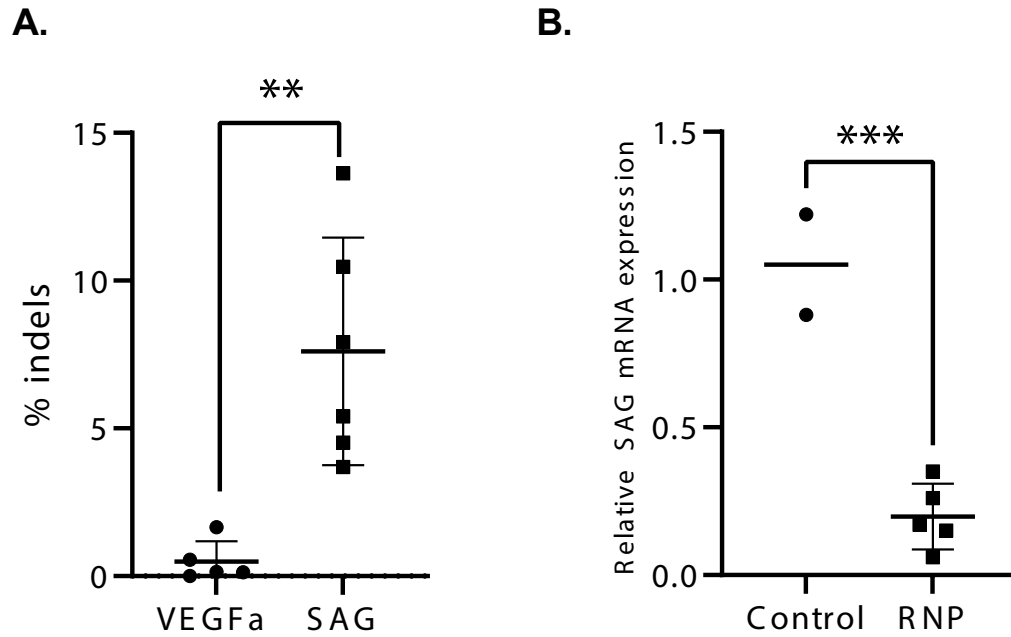


Figure 6: Efficiency of different sgRNA targeting gene highly expressed in the retina.

A/ Frequencies of indels induced in the whole neural retina after 2uL sub-retinal injection of Cas9 RNP (30uM, ratio 1:1) in WT mice. NGS analysis was performed 7 days after transfection. Mean ± SD. Student's t-test: ** $p < 0.005$

B/ SAG mRNA expression level in mouse retinas injected with Cas9 RNPs compared to non-injected eyes. qPCR was performed 7 days after transfection. Mean ± SD. Student's t-test: *** $p < 0.005$.

Discussion

Regardless of the size of the gene or the nature of the mutation, gene editing tools provide unprecedented opportunities for effective and long-lasting treatment of both dominant and recessive forms of vision loss (Anzalone et al. 2019; Saha et al. 2021). In this context, persistent expression of the gene editing protein is undesirable and there is high interest in developing a transient delivery system for this new approach to gene therapy. In the retina, previous works had shown efficient edition of RPE cells *in vivo* after direct delivery of Cas9 RNP complexed with cationic lipids or other synthetic carriers. Intracellular delivery of Cas9 RNP using lipofectamine 2000 lead to therapeutic effects 7 days after injection *in vivo* with potential applications in AMD (E. Kim et al. 2017a). In this study, a 22% indel rate was achieved among the transfected cells 3 days after injection. In a more recent study, a biodegradable nanocapsule (NC) of 25um has been reported to complex and deliver Cas9 RNP to the RPE. NCs were decorated by all-trans-retinal, a coumpound that is naturally transported from the photoreceptors to the RPE cells as part of the visual cycle. It's only with this addition that NC/all-trans-retinal outperformed Cas9 RNP delivery alone (Chen et al. 2019).

In our study, we investigated how to deliver Cas9 as a protein into retinal cells with a particular focus on photoreceptor cells *in vivo*. Direct sub-retinal delivery of Cas9 protein with 2 NLS and a sgRNA is sufficient to edit photoreceptor cells after sub-retinal injection. However, we found that the majority of Cas9 protein is blocked by the outer segments of PRs which seem to obstruct passage of RNPs into the outer nuclear layer. Indeed, photoreceptors form a dense layer of cells that present specific barriers as photoreceptor outer segments and external limiting membrane. The pore size of the interphotoreceptor matrix might pose an additional obstacle to the spread and cellular entry of the RNPs away from the injection site. The size of RNP complexes and their capacity to diffuse are important parameters to understand in order to obtain successful RNP delivery. Particle size restriction might partially explain why we do not observe improvement of indel rates with RNPs delivered with non-viral vectors.

On the contrary to the dense neural retinal tissue, phagocytotic RPE cells form a monolayer giving an intrinsic advantage for macromolecule delivery into these cells. These can explain the higher indel rates that we observed in the RPE after subretinal injections in mice. In addition, big RNP complexes formed with cationic lipids seems to improve delivery into the RPE compared to RNP alone.

Moreover, the addition of peptide SP10 to Cas9 RNP complexes leads to heterogeneous particles that do not improve indel frequencies obtained in the neural retina. Peptides were shown to improve intracellular RNP delivery in the brain (Cas9 protein fused to multiple NLS sequences (Staahl et al. 2017) and *in vitro* (Jain et al. 2019). Other peptide modifications as SP10 peptide could improve complexation, intracellular delivery efficiency, endosomal escape and targeting specificity (Krishnamurthy et al. 2019). A covalent linkage between the peptide and Cas9 protein might facilitate formation of smaller and more homogenous complexes and potentially aid intracellular RNP delivery.

Our results also raised concerns over the validation of sgRNAs using *in vitro* cell lines. Indeed, indels induced in a murine cone cell line by sgRNA targeting *VEGFa* gene were found to be similar to those induced by sgRNA targeting the highly expressed SAG gene. Strikingly, these results were not coherent with *in vivo* studies where sgRNA targeting SAG gene induced 10 times more indels compared to sgRNA targeting *VEGFa* gene. Accessibility of the chromatin to the Cas9 protein is a key parameter that must be considered (Wu et al. 2014). In addition, editing efficiency between cell types or in a context of degenerative diseases is not well understood and more work is needed to understand the contribution of these parameters to the efficacy of gene editing.

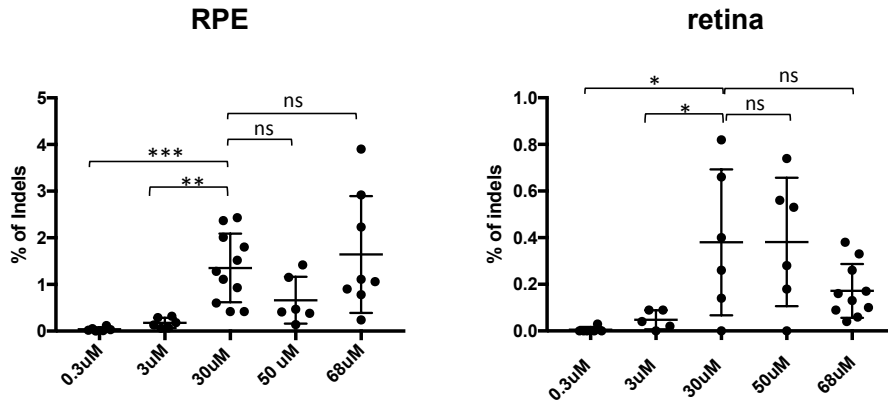
Finally, the percentage of edition needed to have a therapeutic effect will depend on the disease. Maeder and colleagues estimate an editing efficacy of 10% to provide benefit to LCA10 patients affected by mutations in CEP290 (Maeder et al. 2019). When targeting the SAG gene, we were able to reach a photoreceptor indel frequency with a mean at 7.6% that translated to a high decrease in SAG mRNA levels. Additionally, some toxicity and early immune responses were detected one month after RNP injection showing further optimization is needed before we can expect a therapeutic benefit with such an approach. A

decrease of Cas9 concentration and its potential shielding from immune cells in subretinal space is thus needed (Table S2).

In conclusion, our work shows the possibility to transiently deliver Cas9 directly as an RNP into RPE and photoreceptor cells *in vivo* and opens the door for optimization of delivery strategies and use of other gene editing proteins into this tissue.

Supplementary results:

A.



B.

	sgRNA
Reference	GAAGCTACTGCCGTCCGATTGAGAC <u>CC</u> TGGTGGACATCTTCCAGGAGTACCCC
+1	GAAGCTACTGCCGTCCGATTGAGAC <u>CC</u> TGGTGGACATCTTCCAGGAGTACCCC
-1	GAAGCTACTGCCGTCCGATTGAGAC <u>CC</u> TGGT- GACATCTTCCAGGAGTACCCC
-2	GAAGCTACTGCCGTCCGATTGAGAC <u>CC</u> TGG- - GACATCTTCCAGGAGTACCCC
-3	GAAGCTACTGCCGTCCGATTGAGAC <u>CC</u> TGG- - - ACATCTTCCAGGAGTACCCC

Figure S1: Direct RNP delivery induces indels *in vivo*.

A. Percentage of indels in the RPE and neural retina after sub-retinal injection of Cas9 RNP at different concentrations *in vivo*. NGS analysis was performed 7 days after injection. Mean $\pm$ SD. Student's t-test: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.005$

B. Indels profile observed by NGS analysis after Cas9 protein and sgRNA targeting *VEGFa* gene (in blue). PAM sequence is underlined. Indels (in red) occurs at 3pb from the cut.

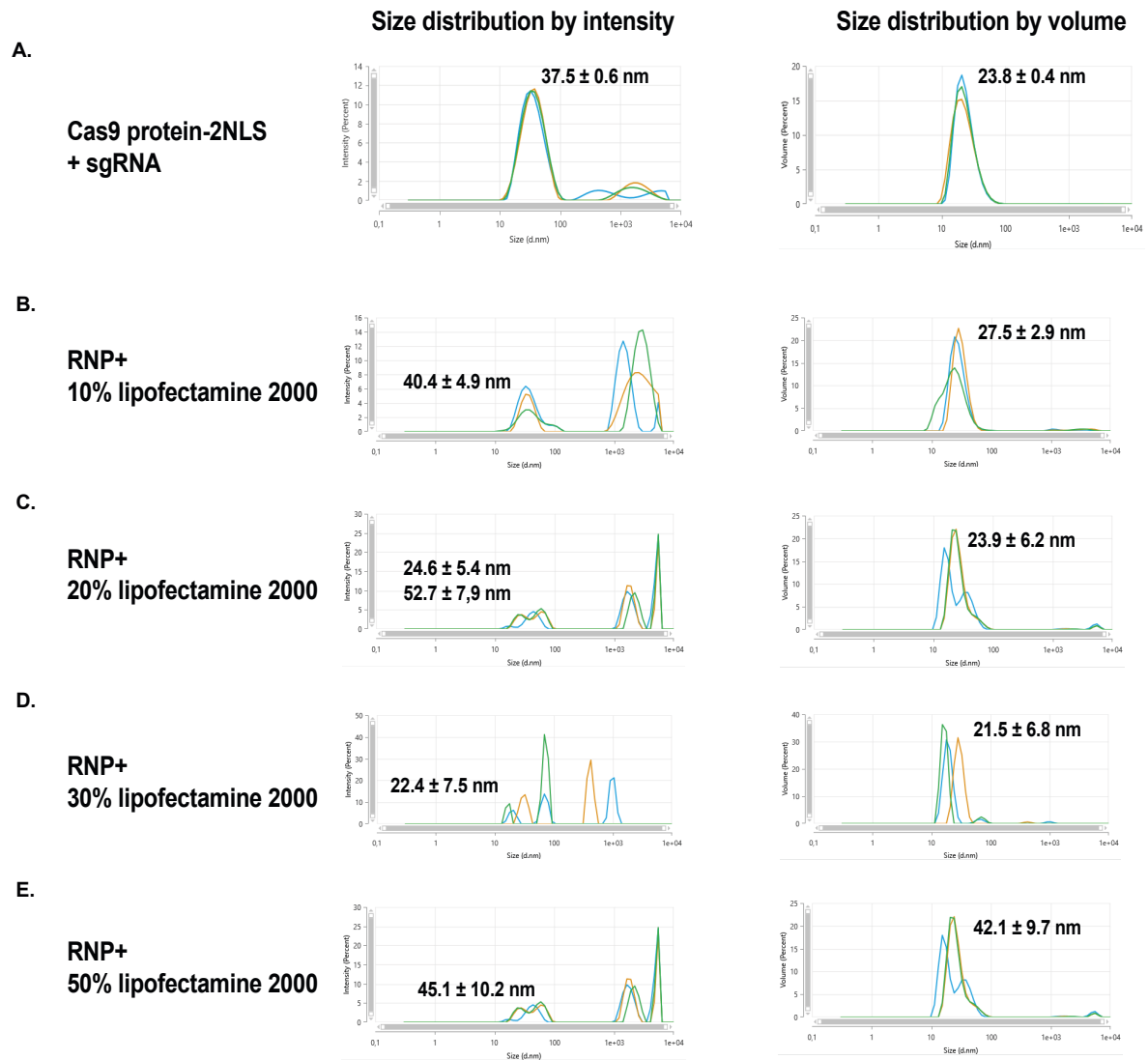


Figure S2: Size determination of Cas9 RNP and Cas9 RNP complexed with lipofectamine 2000.

Size distribution by intensity and volume of Cas9 protein and its sgRNA: (A) without complexation or (B to E) complexed with an increase volume of lipofectamine 2000 (lipids represent from 10% to 50% of total volume of injection). Three different measures (in blue, red and green) were performed at 5-minute intervals.

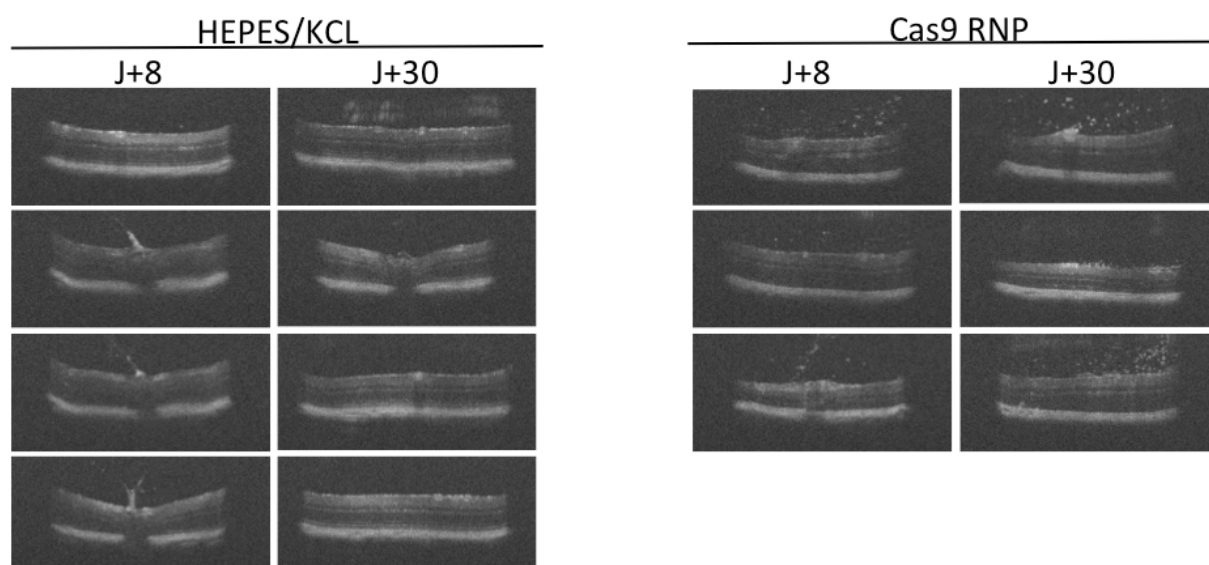


Figure S3: Optical tomography images (OCT) of mice injected by Cas9 RNP at D8 and D30 compared to control mice.

Mice were injected with Cas9 RNP (30uM), Control mice were injected with Buffer solution. Central images performed at D8 and D30 post-injection by OCT.

A.

Mice samples		% indels	Profil
RNP 25uM	1	0	/
	2	0	/
	3	0.35	Insertion 1pb (T) + Deletion 1pb (T or G) + Deletion 3pb (TGG)
	4	0	/
	5	0	/
	6	0.03	Insertion 1pb (T)

B.

Human samples		% indels	Profil
RNP 25uM	1	0.02	Deletion 3pb (TGG)
	2	0	/
	3	0.04	Insertion 1pb (T)
	4	0.04	Insertion 1pb (T)
	5	0	/
	6	0	/

C.

Non-human primate Samples		% indels	Profil
25uM RNP	1	0.12	Insertion 1pb (T)
	2	0	/
	3	0	/
	4	0	/
25uM RNP complexed with cationic lipids	1	0	/
	2	0	/
	3	0	/
25uM RNP complexed with SP10	1	0.02	Insertion 1pb (T)
	2	0.04	Insertion 1pb (T)
	3	0	/

Table S1: Summary of conditions and NGS analysis found in mice, non-human primate and human retinal explants.

Indels induced after transfection of Cas9 RNPs (30uM) alone or complexed with non-viral vectors in A/ mice retinal explants, B/ Human retinal explants and C/non-human primate retinal explants. Explants of 5mm² were placed on a membrane with the photoreceptors up. NGS analysis was performed 7 days after transfection.

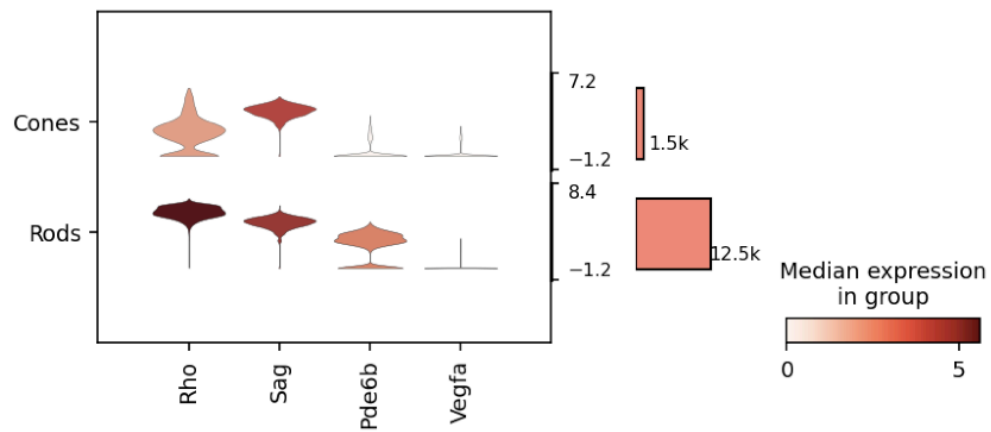


Figure S4: Expression level of *VEGFa*, *PDE6b*, *SAG* and *RHO* gene in cones and rods of wild-type adult mice.

RNAseq analysis based on wild type adult mice database (GSE132229) (Heng et al. 2019).

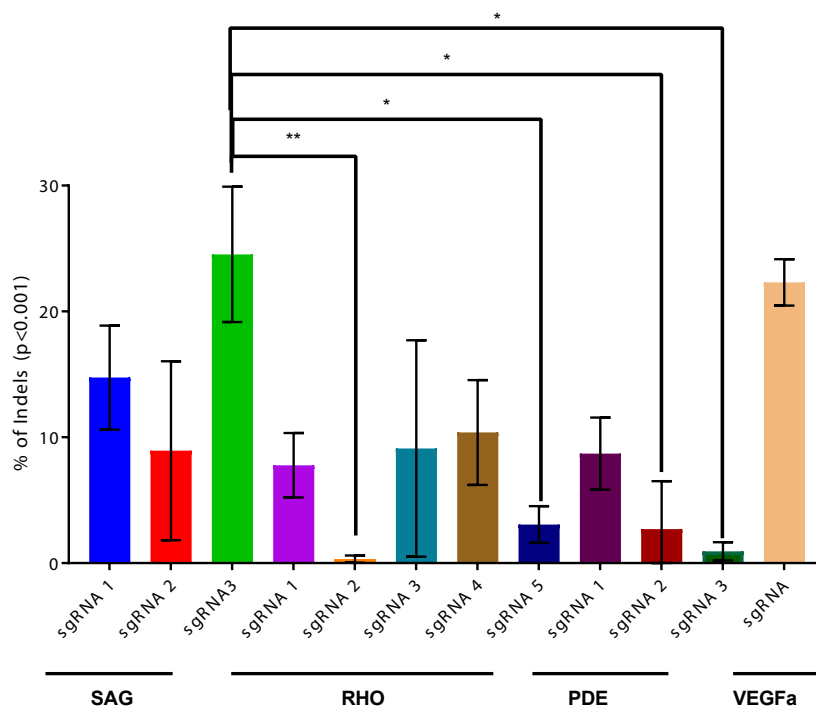


Figure S5: Validation of sgRNAs targeting different genes highly expressed in photoreceptors (SAG, RHO and PDEb) and compared to a sgRNA targeting *VEGFa* gene.

Transfection of Cas9 RNPs complexed with lipofectamine 2000 in 661W cell line. After 48 hours, cells were harvest and percentage of indels was determined by TIDE assay. Transfection experiment was done in triplicate except for *VEGFa* gene (duplicate). Mean $\pm$ SD. Ordinary One-way ANOVA test: * $p<0.05$, ** $p<0.005$. Only significant differences compared to SAG sgRNA3 are shown.

Cas9 protein	Concentration And Volume	Molecules
<i>In vitro</i> ($5 \cdot 10^5$ cells)	100nM in 275uL	$1.7 \cdot 10^{13}$
<i>In vivo</i> ($7 \cdot 10^9$ neural retina cells)	30uM in 2uL	$3.6 \cdot 10^{13}$

Table S2: Calculation of Cas9 molecules transfected *in vitro* or injected in the sub-retinal space *in vivo*.

In vitro, $5 \cdot 10^5$ were transfected by 100nM in a total volume of 275uL. *In vivo*, 2uL of Cas9 RNPs solution at 30uM concentration were sub-retinally injected. Neural retina cells number is estimated at $7 \cdot 10^9$ cells (Jeon et al. 1998). Number of Cas9 molecules is determined based on Avogadro constant ($1 \text{ Na} = 6.02 \cdot 10^{23} \text{ mol}^{-1}$).

Material and Methods

sgRNA design

sgRNAs targeting mouse *PDEb*, *SAG* and *RHO* gene were designed using CRISPOR. sgRNA were design outside of nucleosomes location predicted with <http://dna.engr.latech.edu/icm-du> (Sereda and Bishop 2010; Stolz and Bishop 2010).

The *VEGFa* sgRNA targeting a commun sequence between species of the murine vascular endothelial growth factor A (*VEGFa*) gene was published by Kim and colleagues (Kim et al., 2017). Sequences of 20pb variable of sgRNAs used in this study are listed in supplementary Table S3.

All sgRNA were synthesized and purified using the GeneArtTM Precision gRNA Synthesis Kit (Invitrogen), according to the manufacturer's protocol. sgRNA eluted in water were aliquoted and stored at -80°C.

SpCas9 proteins

SpCas9 protein with 2 nuclear localization sequences (NLS) (one on its N and one on its C terminal) and SpCas9 protein with 6 NLS (2NLS on its N and 4NLS on its C terminal) were produced and given by Laboratoire Structure et Instabilité des Génomes (MNHN Paris – Jean Paul Concordet). For *in vitro* analysis, SpCas9 protein, which contains two nuclear localization sequences, was purchased from Aldevron.

661W cell culture

Cone murine 661W cell line (ATCC, Virginia, USA) was cultivated in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) complemented with 10% (v/v) fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco). Cells were maintained in a 37°C, 5% CO₂, fully humidified incubator, and passaged twice weekly.

RNP preparation and complexation

Ribonucleoproteins (RNP) were prepared immediately before used. First, synthesized sgRNA is diluted in a 40mM HEPES/551mM KCl buffer (pH7.4) to obtain a solution at 20mM HEPES/200mM KCL. sgRNA is further diluted in a 20mM HEPES/200mM KCl (pH7.4) to have a constant final volume of RNP solution. Then SpCas9 protein is added to obtain a ratio Cas9

protein:sgRNA of 1:1. RNP solution is incubated at room temperature for 5 minutes before direct use or complexation with a vector.

Different non-viral vectors and compounds were mixed with RNP, vortex for 10 seconds and incubated at room temperature 10min for characterization or until injection. Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen) were purchased from ThermoFischer. Shuttle peptides were synthesized by Covalab.

Dynamic Light Scattering

The hydrodynamic diameter (size) of each complex was measured using DLS (Zetasizer nano analyser ZS; Malvern Instruments) at Paris ESPCI facility. Three different replicates were carried out with 5 minutes in between each measure.

Electronic microscopy

Samples were prepared and visualized by electronic microscopy at Faculté de Pharmacie de Paris. Briefly, samples were placed on carbon-coated grids and negatively stained with 2% uranyl acetate for 4 minutes.

***In vitro* transfection in 661W cell line**

661W were plated at 5×10^4 cells per well in a 48-well plate with a total volume 250 μ L/well. 24h after plating, cells (70-90% confluency) were transfected with Lipofectamine 2000 or RNAiMAX following the manufacturer's protocol. Briefly, sgRNA and spCas9 were mixed by pipetting 4 times, and incubated at room temperature for 5 minutes.

In parallel, 2 μ L of cationic lipids (Lipofectamine™ 2000 or Lipofectamine®RNAiMAX from Invitrogen) were added to a final volume of 25 μ L of Opti-MEM™ Reduced Serum Medium (ThermoFisher). Then Cas RNP was added to Opti-MEM/lipid solution, vortexed for 10 seconds and incubated at room temperature for 10 minutes. 25 μ L of complexed solution was added to each well to obtain a final RNP concentration of 100nM in a total volume of 275 μ L. Medium was change 24h after transfection and cells were harvested 48 hours after transfection. Cell pellet was washed with PBS and frozen at -20°C until DNA extraction. Genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel).

Subretinal injections in mice

For animals, the experiments were realized in accordance with the NIH Guide for Care and Use of Laboratory Animals (National Academies Press, 2011). The protocols were approved by the Local Animal Ethics Committees and conducted in accordance with Directive 2010/63/EU of the European Parliament.

C57BL/6j wild type (Janvier Labs) and C57BL/6jrd10/rd10 (rd10) mice (breeding done at Institut de la Vision) were used for this study. rd10 mice have a mutation on the rod PDE gene leading to a rod-cone dystrophy. For ocular injections, mice were anesthetized by isoflurane inhalation. Pupils were dilated and sub-retinal injections of 2 uL were performed using a Nanofil syringe with a 28G blunt needle (World Precision Instruments, Inc.) under an operating microscope (Leica Microsystems, Ltd.). Ophthalmic ointment (Fradexam) was applied after surgery. Eyes with extensive subretinal hemorrhage were excluded from the analysis.

Animals were euthanized by CO₂ inhalation and cervical dislocation. After 3 days, 7 days or 1 month, the whole RPE and the whole retina were isolated without selection of transfected cells. Optical coherence tomography (OCT) images were taken 1 month after injection under isoflurane anesthesia. OCT images were analysed to measure the thickness of each retina layers by InvivoVue Diver software (Fig. S6).

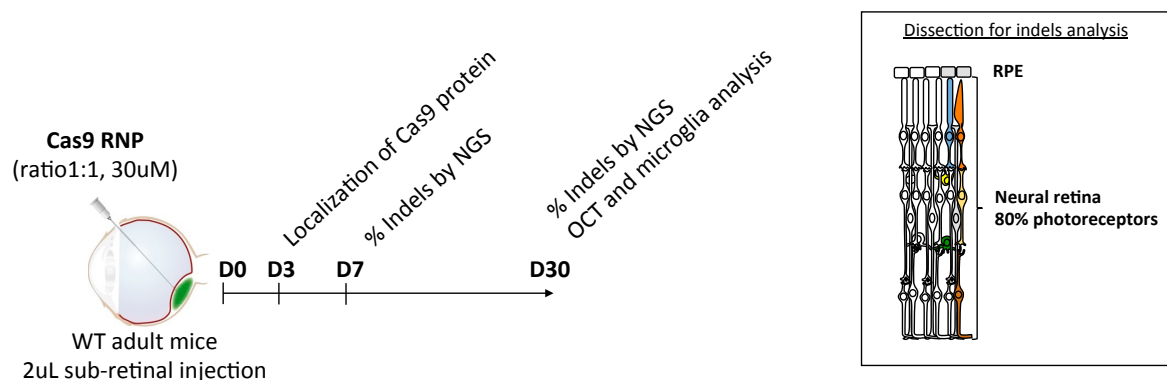


Figure S6: General protocol for *in vivo* study.

Cas9 protein staining was performed at D3 post injection. Toxicity studies by OCT and Ibal staining were performed at D30. RPE and Neural retina composed of 80% of photoreceptors in WT mice were isolated at D7 and D30 to analyse indels induced by Cas9 RNP.

Dissection of human and macaque eye

The human eyes were obtained through Dr. Djamel TALEB thanks to people who gave their bodies to science and according to an agreement between Dr. Deniz DALKARA, Dr. Serge PICAUD, and Pr. P. FRILEUX, scientific director of the school of surgery of the Pitié-Salpêtrière. They were transported according to the regulations for the transport of infectious substances (based on the practical guide published by the WHO, n°WHO/HSE/EPR/2008.10 in force on January 1, 2009) of category B assigned to UN N° 3373. The *macaca mulatta* eyes were obtained from monkeys already under experimental procedures at MIRCEN.

The eyes were transported in CO₂-independent medium (Life Technologies). The eye was first rinsed in two baths of Pursept® (Sigma) and one bath of CO₂-independent medium + 0.1% gentamycin. Excess tissue is removed, then the eye is pierced with an 18 gauge needle (Microlance™) approximately 5mm below the ora serata to allow removal of the cornea and lens. The RPE is gently separated from the choroid down to the optic nerve. The retina and RPE are then placed in CO₂-independent medium + 0.1% gentamycin. The retina is gently separated from the RPE and the vitreous is then removed (Fradot et al. 2011). Retinal explants of 5mm² are cut and placed on a moistened membrane (Costar) with the

photoreceptors cells side up. The wells are filled with 2mL of Neurobasal medium + 0.1% gentamycin + 1% Glutamine + 2% B27 (Life Technologies). One drops (2 μ L) of transfected solution are placed on the center of the explants. The explants were then maintained in a 37°C, 5% CO₂ for 7 days.

Immunostaining and imaging

Three days post-injection, mouse eyes were enucleated and immediately fixed in 10% formalin/4% formaldehyde for 1 hour. Retinal explants were first washed with PBS then fixed with the same procedure.

To prepare cryosections, eyecups and explants were immersed in PBS-10% sucrose for 1 hour and then PBS-30% sucrose overnight at 4°C. They were embedded in OCT medium and frozen in liquid nitrogen. 12 μ m-thick vertical sections were cut with a Microm cryostat.

After 3 PBS washes, retinal flatmounts or cryosections were incubated in a blocking buffer for 1 hour and then with primary antibodies overnight at 4°C. Primary antibodies used in this study are listed in Supplementary table S6. After three washes of the sections, the secondary antibodies (Alexa Fluor 488, 594 or 647, Thermo Fischer Scientific) were added for 2 hours at room temperature, followed by three PBS washes. Retinal flatmounts or cryosections were mounted in Vectashield mounting medium (Vector Laboratories) and visualized using an Olympus confocal microscope. ImageJ software was used to process the images.

Samples preparation for quantification of Indels

For genomic DNA extraction, samples from each experimental condition were incubated in lysis buffer and proteinase K at 56°C overnight for retina samples, 3 hours for RPE tissue and 10min for *in vitro* cells. Further steps were performed following the manufacturer's instructions (NucleoSpin® DNA tissue, MACHEREY-NAGEL). We used the GXL HiFi DNA polymerase (Takara) for PCR amplification. Primers for amplifying region of interest are listed in supplementary table S4. The thermal cycler program for PCR was as follows: 98° C for 10s, followed by 60° C for 15 s and finally 68° C for 20s, with in total 30 cycles. PCR samples were then purified using following the manufacturer's instructions (NucleoSpin® PCR and gel kit, MACHEREY-NAGEL). Purified amplicons were verified by electrophoresis on 1% agarose gels.

For *in vivo* and *ex vivo* experiments, PCR amplicons were sent to next generation sequencing

(NGS) at Massachusetts General Hospital DNA core facility. A total of 10 000 reads were generated per samples and analysis were done with a cut off of 10 reads. For *in vitro* experiments, PCR amplicons were sent to Sanger sequencing (Eurofins) and indels were analysed using TIDE (Tracking of Indels by Decomposition) Analysis (<https://tide.deskgen.com/>). Analysis was done using a reference sequence from untreated samples and setting the parameters to detect a maximum Indel size of 10 nucleotides.

qPCR analysis

After 7 days post-injection, retinas were collected from each experimental condition (n = 4-5 retinas). Total RNA was harvested using a RNA-extraction kit (NucleoSpin RNA, Macherey-Nagel), follow by a DNase digestion step with Turbo DNase (ThermoFischer Scientific). Reverse transcription was performed with Superscript Reverse Transcriptase III, following the manufacturer's instructions (ThermoFischer Scientific) with oligodT primers (ThermoFischer Scientific). For qPCR, samples were run at triplicates with no-RT controls to confirm the absence of genomic DNA. Primers used are listed in supplementary table S5. cDNA levels were determined with relative cDNA quantification and are expressed as the fold induction compared to control groups (non-injected animals).

Statistical analysis

All statistical analyses were carried out using GraphPad PRISM version 7.0. Error bars on the graphs show values $\pm$ SD. P-values were determined by non-parametric student t-test.

Gene (exon) targeted	sgRNA name	Sequence
VEGFa (exon3)	VEGFa sgRNA	CTCCTGGAAGATGTCCACCA
RHO (exon 1)	RHO sgRNA 1	GGTTCCGCCAGGTAGTACTG
	RHO sgRNA 2	TAGAGCGTGAGGAAGTTGAT
	RHO sgRNA 3	GCCAGCATGGAGAACTGCCA
RHO (exon 5)	RHO sgRNA 4	CAAGACGGAGACCAGCCAGG
	RHO sgRNA 5	GAGGCGTCGTCATCTCCCAG
PDE (exon 13)	PDE sgRNA 1	GCCGTGGCGCCAGTTGTGGT
	PDE sgRNA 2	GAGTAGGGTAAACATGGTCT
	PDE sgRNA 3	GTGGTAGGTGATTCTTCGAT
SAG (exon 2)	SAG sgRNA 1	TCCCTTGGGATGTACGTCAG
	SAG sgRNA 2	GACCTTCTTGAAGATGACGT
SAG (exon 8)	SAG sgRNA 3	AGACATGAAGAACTGCCAGG

Table S3: sgRNA sequences used in this study.

Sanger Primers		Sequence
VEGFa exon 3	Fw	CAAATCTGGGTGGCGATAGA
	Rev	AGATGGTCAAATCGTGGAGAG
RHO exon 1	Fw	ATATCTCGCGGATGCTGAAT
	Rev	GCAAAGAAGCCCTCGAGATT
RHO exon 5	Fw	CATGTGGACTTCGGTTTTCC
	Rev	GGAGCCTCATTTTGCTTTCA
PDE exon 13	Fw	GGCCAGTGAGAACAAGGAAC
	Rev	GCTCCAGAAGGCAGTGGTTA
SAG exon 2	Fw	GCTGCACACTACAGTAAAGTACAACTG
	Rev	TCCACTGTTGTTCTTGGTTC
SAG exon 8	Fw	GGTATCTGAAACCATGACACAGA
	Rev	GGGGAGAGCCAACAAGTACA
NGS Primers		Sequence
VEGFa exon 3	Fw	TCATGGATGTCTACCAGCGA
	Rev	AATGCCCCTCCTTGACCAC
SAG exon 8	Fw	GCTCTGTGCGGTTACTGATC
	Rev	AGTGGAATAAGCTGGTAATTTGCA

Table S4: Primers used to amplify the targeted regions of each gene for Sanger or NGS sequencing.

qPCR Primers		Sequence
Actin	Fw	GTGGTACGACCAGAGGCATAC
	Rev	AAGGCCAACGCTGAAAAGAT-
SAG exon 8	Fw	GACAAGCCCCTGAACCTCTC
	Rev	TGTTGTTGGTCACGGTCACA

Table S5: Primers used for qPCR analysis.

Antigen	Species	Dilution	Source
IBA1	Rabbit polyclonal	1/250	WAKO
RECOVERIN	Rabbit polyclonal	1/1000	Merck/Millipore
RHODOPSIN	Mouse monoclonal	1/1000	Merck/Millipore
SpCAS9	Mouse monoclonal	1/1000	Genscript
ZO-1	Rabbit polyclonal	1/250	ThermoFischer Sci.

Table S6: Primary antibodies used for immunostaining.

Acknowledgements:

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Additional results

I. Additional results on Cas9 protein delivery

1. Cas9 protein and its buffer optimization for *in vivo* injection

Cas9 protein and its buffer are key parameters to avoid precipitation of newly formed RNP complexes and ensure RNP efficacy *in vivo*. We first compare *in vivo* our SpCas9-2NLS protein produced in Muséum national d'histoire naturelle (MNHN) to a commercialized SpCas9 protein with 2 NLS from Aldevron compagny. Each Cas9 protein were incubated with an sgRNA targeting *VEGFa* gene and injected in the subretinal space of WT mice. Cas9 protein from MNHN induced higher indels compared to Aldevron proteins 7 days post-injection and was kept for further *in vivo* studies (Fig. S7).

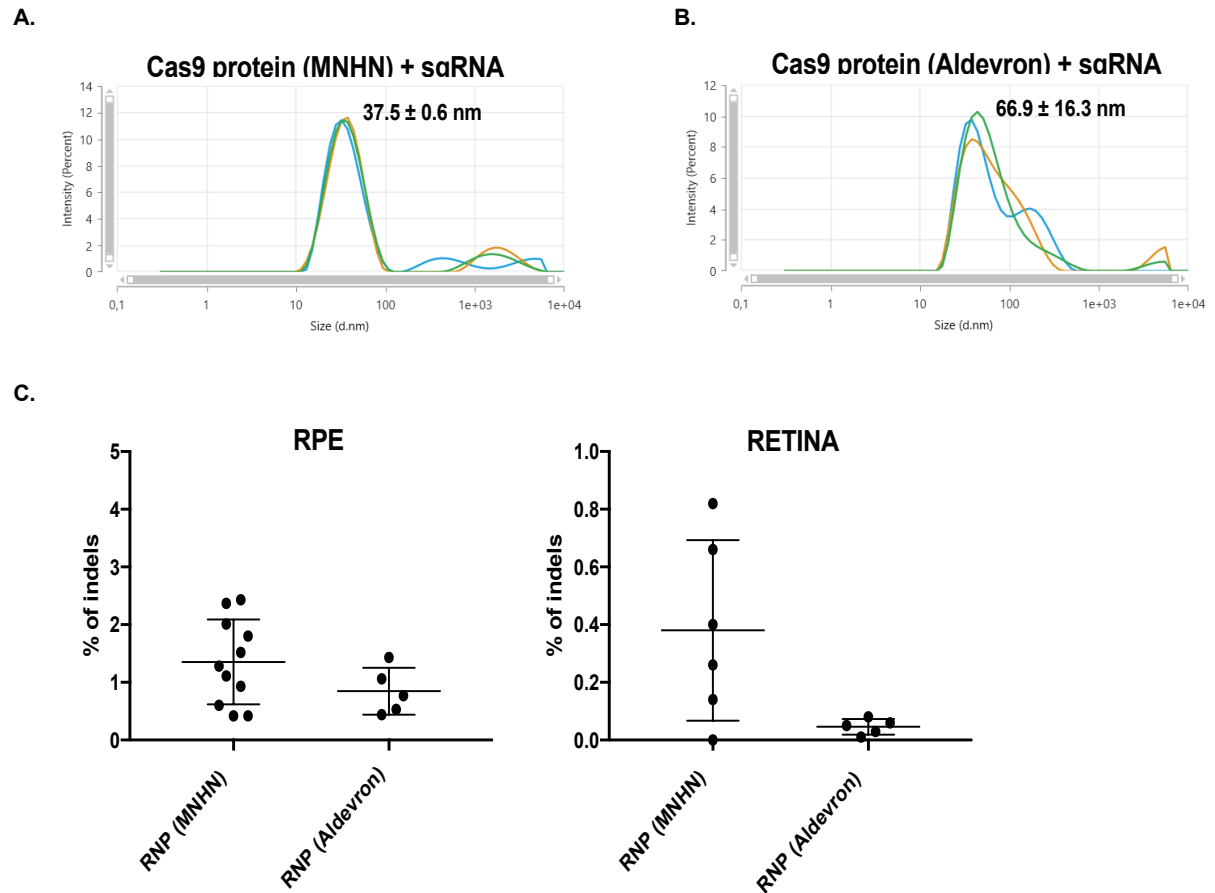


Figure S7: Comparison of different Cas9 proteins and different buffers *in vivo*.

Size distribution by intensity of sgRNA complexed to (A) Cas9 protein from MNHN, (B) Cas9 protein from Aldevron. Three different measures (in blue, red and green) were performed at 5-minute intervals.

C. Indels analysis in the RPE and neural retina after sub-retinal injection in wild-type mice of 2 μ L RNPs (30 μ M) with Cas9 protein produced by MNHN compared to a commercialized protein from Aldevron company. sgRNA used in this experiment targets *VEGF α* gene. NGS analysis was performed 7 days after injection. Mean $\pm$ SD. Student's t-test: * $p < 0.05$.

2. Volume of RNP injection in sub-retinal space

Volume of injection in sub-retinal space is a critical parameter in view of toxicity. We compared a 2uL injection of Cas9 RNP to a 1uL injection, frequently used in the context of AAV injection in mice. After 7 days, no significant differences of indels rate in the RPE and the neural retina were observed between the two conditions albeit with a higher indel rate after a 2uL injection (Fig. S8).

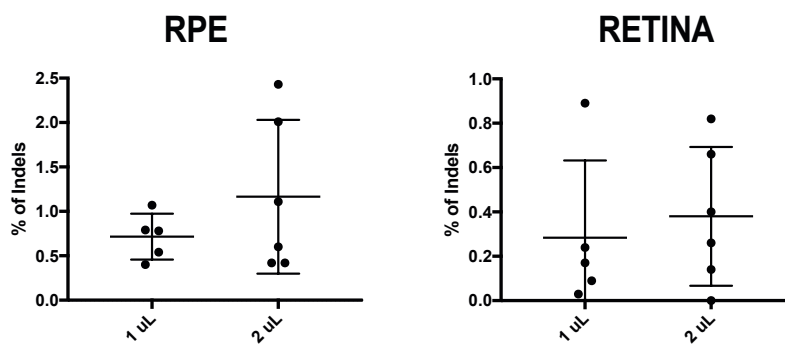


Figure S8 : Indels efficiency after 1uL or 2uL of RNPs injection.

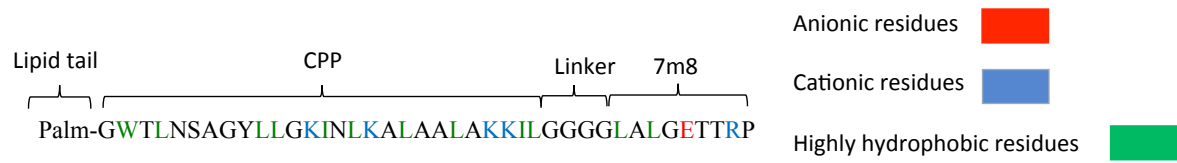
Indels analysis in the RPE and neural retina after 1 or 2uL of Cas9 RNPs sub-retinal injection in wild-type adult mice. NGS analysis was performed 7 days after injection. Mean $\pm$ SD. Student's t-test: ns: non-significant

3. Non-viral vectors to deliver Cas RNPs *in vivo*.

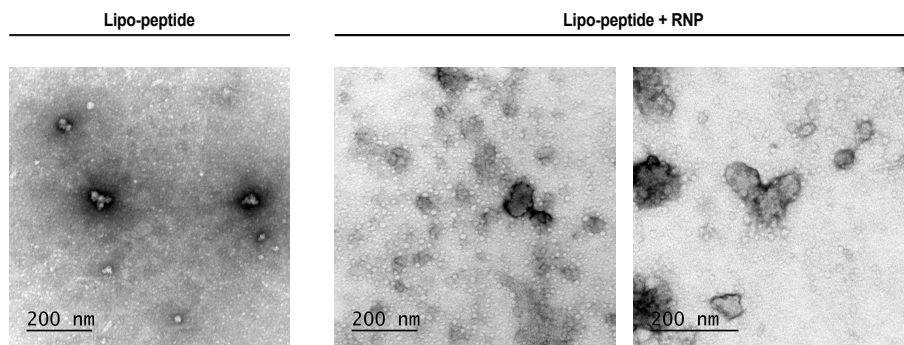
3.1 A lipopeptide to complex and deliver RNP to photoreceptors.

A lipid tail linked to a tandem peptide carrying a net positive charge showed capacity to efficiently complex RNPs and deliver them to several cell types (Jain et al. 2019). The tandem peptide was composed of a cell penetrating peptide (CPPs) and a targeting peptide. Based on this study, we engineered a lipo-peptide composed of a lipid tail fused to a modified tandem peptide (Sequence: Palm-GWTLNSAGYLLGKINLKALAALAKKILGGGGLALGETTRP, net charge +4). Here, we replaced the tumor targeting sequence used by Jain and coworkers with a short sequence LALGETTRP named 7m8 (Fig. S9A). The 7m8 sequence was obtained through a directed evolution screen of AAVs to target photoreceptor cells *in vivo* (Dalkara et al. 2013). Complexes of 208 ± 47.1 nm were observed in DLS and electronic microscopy and injected sub-retinally to WT mice (Fig. S9B). At a concentration of 25uM, RNP delivery by lipo-peptide vector induced 0.41 to 3.35% indels in the RPE and up to 0.14% in the neural retina. (Fig. S9C). Similar results were obtained after transfection of RNP complexed with lipo-peptide in retinal explants of human and non-human primate (Table S7). Taken together, these results showed that our lipo-peptide is not able to deliver efficiently Cas9 RNP inside the neural retina.

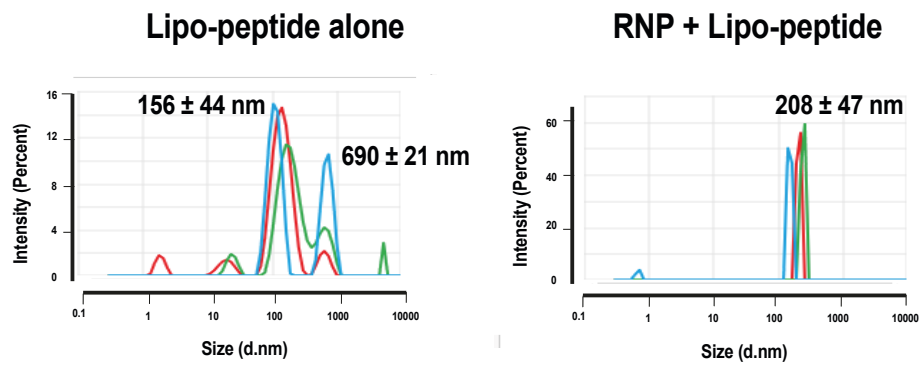
A.



B.



C.



D.

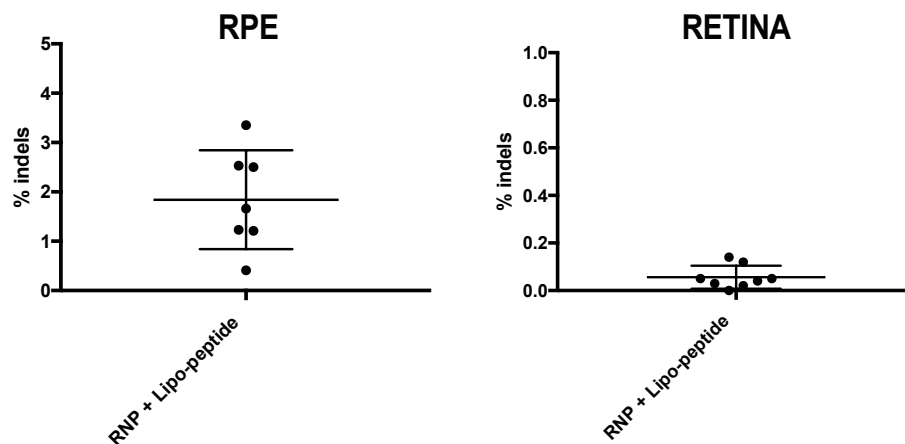


Figure S9: Design, size characterization and *in vivo* efficiency of RNP delivery with lipo-peptide vector.

- A. Lipo-peptide composed of a lipid tail, a CPP and 7m8 peptide. Anionic amino acids are highlighted in red; cationic amino acids are in blue and highly hydrophobic amino acids are in green.
- B. Cas9-2NLS RNP (6uM) complexed to lipo-peptide negatively stained with 2% uranyl acetate for 4 minutes and observed by electronic microscopy.
- C. Size of Cas9-2NLS RNP (30 uM, ratio 1:1) complexed with lipo-peptide determined by DLS. Three different measures (in blue, red and green) were performed at 5-minute intervals
- D. Indels rate induced in the whole RPE and retina after sub-retinal injection of Cas9 RNP (25uM or 6uM) complexed with lipo-peptides *in vivo*. NGS analysis was performed 7 days after injection. Mean $\pm$ SD.

Samples			% indels	Profil
Non-human primate	25uM	1	0.04	Insertion 1pb (T)
		2	0.02	Insertion 1pb (T)
		3	0	/
		4	0	/
Human	25uM	1	0.11	Insertion 1pb (T) + Deletion 3pb (TGG)
		2	0.04	Insertion 1pb (T)
		3	0	/
	6uM	1	0	/
		2	0	/
		3	0.02	Insertion 1pb (T)

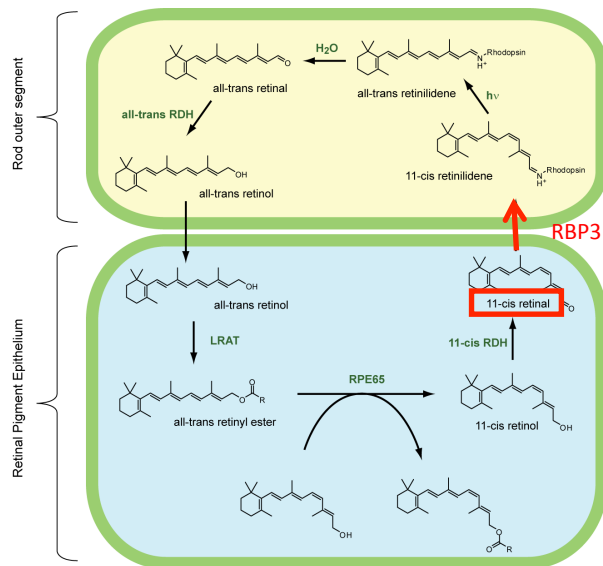
Table S7: Efficiency delivery of RNP complexed with lipo-peptide in non-human primate and human retinal explants

Indels induced after transfection of Cas9 RNPs (6uM and 25uM) complexed with lipo-peptide vector in non-human primate and human retinal explants. Explants of 5mm² were placed on a membrane with the photoreceptors up. NGS analysis was performed 7 days after transfection.

3.2 9-cis-retinal and RNP delivery to photoreceptors

A recent study reported a new carrier to deliver RNPs inside the RPE cells. To improve RNPs delivery, they linked covalently vectors containing RNPs to chemical compounds all-trans-retinal. Indeed, all-trans-retinal is part of the visual cycle and is transported from the photoreceptors outer segments to the RPE cells by RBP3 transporter (Fig. S10A). This addition increases RNPs delivery inside RPE cells. Based on this study, we hypothesized that 9-cis-retinal, derivative of 11-cis-retinal visual pigment that is transported from RPE to photoreceptors by RBP3, could optimize the delivery to PRs (Fig. S10A). We mixed RNPs to 9-cis-retinal compound at three different concentrations and performed sub-retinal injection in WT mice. Compared to RNPs injected alone, no improvement was observed in RNPs mixed with 9-cis-retinal (Fig. 10B). This result suggests that presence of 9-cis-retinal in the sub retinal space does not increase the uptake of the RNPs that surround them. Covalent bond between Cas9 protein and 9-cis-retinal compounds could help to benefit from the high intracellular transport of visual pigments in PRs.

A.



B.

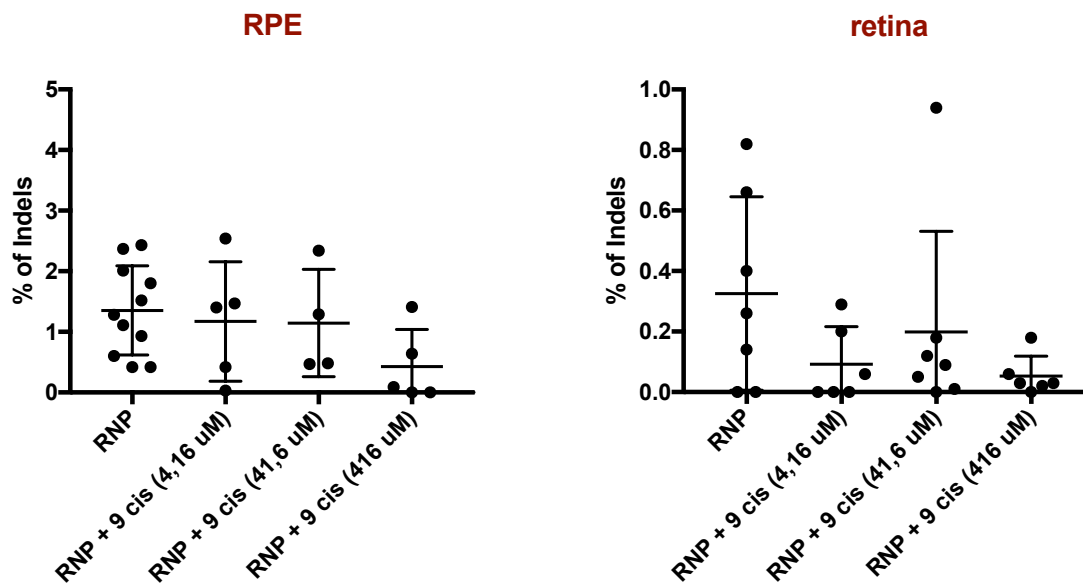


Figure S10: Direct RNP delivery induces indels *in vivo*.

A/ Scheme of the visual cycle. Retinol binding protein (RBP3) present in the extracellular matrix transports visual pigments. All-trans-retinal is transported from the outer segment discs of PRs to the RPE. 11-cis-retinal is transported from the RPE to the PRs.

B/ Indels analysis in the RPE and the neural retina of wild-type mice after injection of RNPs alone or mixed with 9-cis-retinal. The 9-cis-retinal was diluted in PBS (1X) at three concentrations (4uM, 40uM and 400uM). NGS analysis was performed 7 days after injection. Mean $\pm$ SD.

MATERIELS AND METHODS for additional results

RNP complexation

RNP (ratio 1:1, at 30uM and 6uM) were mixed to lipo-peptide or 9-cis-retinal compounds, vortex for 10 seconds and incubated at room temperature 10min for characterization or until injection. Lipo-peptide was purchased from CPC scientific and resuspended in water. 9-cis-retinal compound was purchased from Sigma and resuspended in 200uL of ethanol before several dilutions in PBS to prepare solutions of 4uM, 40uM and 400uM.

Chapter 2: Cas mRNA delivery to the retina

Introduction

Genome editing derived from CRISPR Cas system has emerged to be a unique strategy to ablate or edit permanently genes and has potential to treat inherited retinal degenerations (IRD). In addition to Cas9, new generations of editors as base editors and prime editors are able to correct precisely and with greater specificity point mutations or small DNA sequence (Anzalone et al. 2019; Gaudelli et al. 2017; Komor et al. 2016). Today, AAV vector is the most used delivery system to edit retinal cells and is used in the first *in vivo* gene editing clinical trial to correct Leber Congenital Amaurosis type-10 (AGN-151587). However, Cas DNA transfer leads to the unnecessary long-term expression of the Cas protein that can increase potential off target effects, vector integration into the genome (Anzalone et al. 2020) and immune responses (Charlesworth et al. 2019). In addition, base and prime editors can lead to bystander effects in the targeted gene in their activity window. Delivery of these new tools is also challenging in photoreceptors, as they do not fit into one AAV.

Optimization of delivery systems is necessary to apply safely and efficiently new gene editing tools in the retina *in vivo*. Genome editors are composed of two to three distinct proteins and are not easy to produce in order to be used as RNPs. On the contrary, mRNA delivery is a highly modular alternative that can overcome limiting carrying capacity of AAV and ensure a transient delivery of gene editing tools (Gillmore et al. 2021). In recent years the emergence of *in vitro* transcribed mRNA has expanded our ability to achieve high gene expression from mRNA by preventing immune response activation in addition to stabilizing the mRNA to expand its half-life (Kowalski et al. 2019).

Despite these exciting developments, there have not been any demonstrations of the application of these technologies in the retina. In the retina, nanoparticle-based nucleic acid

delivery has focused on DNA delivery over the past decades (Battaglia et al. 2016; Han et al. 2012). However, DNA delivery using nanoparticles is inherently inefficient due to the nuclear entry requirement in post-mitotic retinal cells. In contrast, few studies just recently showed reporter gene mRNA delivery to photoreceptors *in vivo* (Devoldere et al. 2019; Patel et al. 2019; Ryals et al. 2020). Patel et al used an ionizable lipid to deliver mRNA in the retina and showing high protein expression in the RPE (Patel et al. 2019; Ryals et al. 2020) raising hope that such mechanism may be useful to deliver mRNA of gene editing tools.

Here, we tested Cas9 mRNA and its sgRNA delivery *in vivo* in mouse photoreceptors. As a first step, we investigate different commercially available cationic lipids to complex and deliver Cas9 mRNA *in vivo* in the RPE and the neural retina of WT mice.

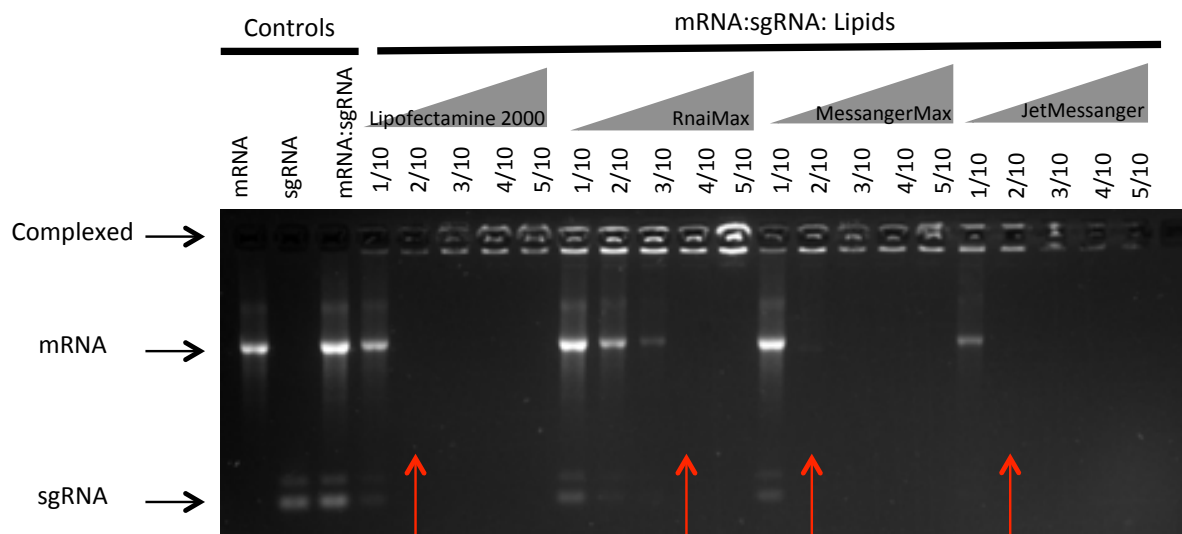
Results

Characterization of SpCas9 mRNA and its sgRNA complexed with cationic lipids

Lipid vectors remain one of the most advanced vectors to deliver mRNA and several lipids are available for *in vitro* and *in vivo* applications. Here, we investigated the capacity of commercially available lipids to complex Cas9 mRNA and sgRNA for an *in vivo* 2uL sub retinal injection application. First, we determined with a gel retardation assay the ability of each lipid to complex Cas9 mRNA and its sgRNA. Lipids for *in vivo* applications, Invivolipofectamine 3.0 and InvivoJetRNA show a high capacity of complexation with the best ratio of 1ug of RNA for 1uL of lipids (Fig. 1B). In contrast, lipids for *in vitro* applications such as lipofectamine 2000, MessengerMax and JetMessenger, complex only 0.4ug of RNA for 1uL of lipid (Fig. 1A). RNAiMAX, a lipid for *in vitro* siRNA applications, has a low capacity of complexation with a ratio of 0.2ug for 1uL of lipids.

We analyzed the size of each complex by dynamic light scattering. Cas9 mRNA with its sgRNA was complexed with lipids leading to heterogeneous particles from 424 ± 78.2 nm (MessengerMax) to 1190 ± 474 (RNAiMAX) in size. Only jetMessenger was able to form monodisperse complexes of 244 ± 12.6 nm with Cas9 mRNA (Fig. 2). InvivojetRNA complexed with Cas9 mRNA and its sgRNA forms complexes with monodisperse size 645 ± 171 nm (Fig. 2). In contrast, Invivolipofectamine 3.0 complexes have heterogenous size with a mean at 473 ± 18.3 nm (Fig. 2). Taken together, Invivolipofectamine 3.0 and InvivoJetRNA show best capacity of complexation of Cas9 mRNA and sgRNA and thus our *in vivo* tests were focused on these two candidates.

A.



B.

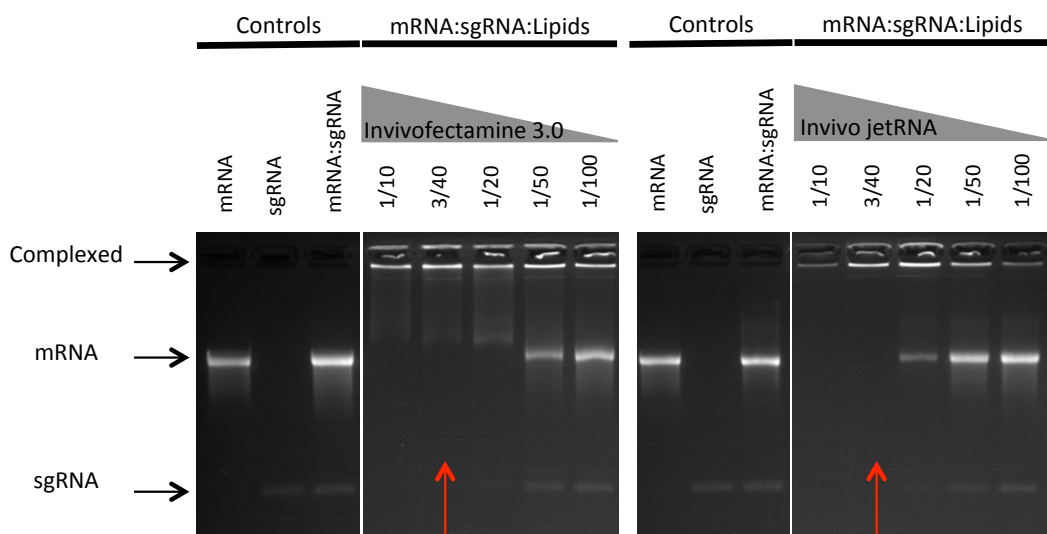


Figure 1: Determination of the best complexation ratio between Cas9 mRNA:sgRNA and lipids.

A. Gel retardation assay of Cas9 mRNA:sgRNA (ratio 1:10) complexed with lipids commercially available for *in vitro* transfection: Lipofectamine 2000, RNAiMAX, MessengerMax and JetMessenger. Prior complexation, lipids were diluted: 1/10 to 1/2.

B. Gel retardation assay for Cas9 mRNA:sgRNA (ratio 1:10) complexed with lipids Invivojectamine3.0 or InvivojetRNA. Prior complexation, lipids were diluted in their specific buffer. Agarose gel (1.5%) was run 20min at 170V. Cas9 mRNA and sgRNA complexed with each lipid stay in agarose well as indicated. Arrows in red show best complexation for each lipid.

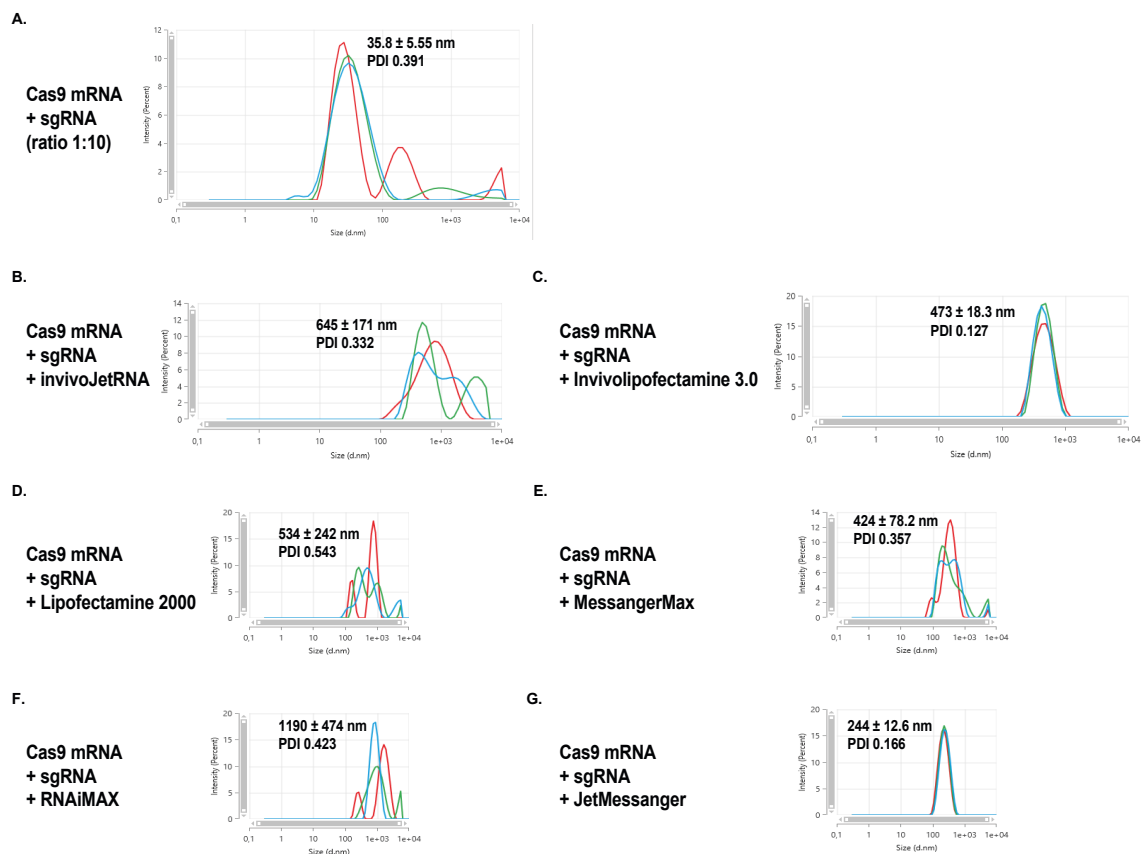


Figure 2: Size measurement of Cas9 mRNA and its sgRNA complexed with different lipids.

Size of mRNA:sgRNA without lipids (A), complexed with InvivojetRNA (B), Invivolipofectamine 3.0 (C), Lipofectamine 2000 (D), MessengerMax (E), RNAiMAX (F) and JetMessenger (G) determined by DLS. Cas9 mRNA and ist sgRNA were mix by vortexing with each lipids and incubated 10 min prior measurement. PDI: Polydispersity index.

SpCas9 mRNA and its sgRNA complexed with cationic lipids do not induce any indels in the neuroretina and few in the RPE.

To target preferentially photoreceptors, we performed sub-retinal injections in adult wild-type (WT) mice of Cas mRNA and a specific sgRNA complexed with Invivolipofectamine 3.0 or InvivoJetRNA with the best ratio of 1ug for 1uL of lipid determined by gel retardation assay. One week after injection, the whole RPE and the whole neural retina were isolated without any cell selection and indels at the target site were quantified by NGS. Indels at mean of 0.5% were found in the RPE and no indels were detected in the neural retina (Fig. 3). In this study, we used the sgRNA targeting *VEGFa* gene. The profile of indels observed was similar to the profile found after Cas RNP injection with an insertion of a T 3pb after the PAM sequence, which validates the low indels rate observed.

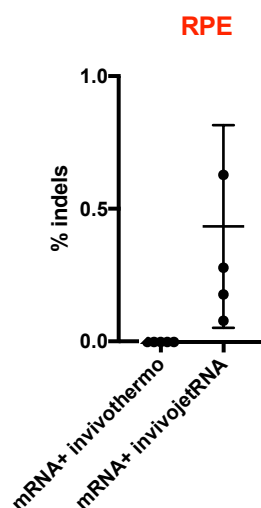


Figure 3: Indels induced after sub-retinal injection of mRNA:sgRNA complexed with lipids *in vivo*.

Percentage of indels in the whole RPE after injection of Cas9 mRNA:sgRNA complexed with Invivofectamine3.0 or InvivojetRNA in WT mice. NGS analysis was performed 7 days after injection. Error bars are SEM. No indels were found in the whole neural retina.

Discussion

mRNA delivery represents a new opportunity to deliver gene editing tools. A first clinical trial reported successful SpCas9 mRNA delivery in the liver thanks to lipid nanoparticles (LNP) modified to be able to package large SpCas9 mRNA (Gillmore et al. 2021). In the retina, first proof of concept reported the delivery of mRNA and expression of reporter proteins in photoreceptors (Devoldere et al. 2019; Patel et al. 2019). Patel et al have screened several cationic or ionizable lipids and showed their capacity to complex mRNA with ionizable lipids to form particles of 80 nm, similar to lentivirus in size, that lead to high protein expression in the RPE (Patel et al. 2019). However, Cas mRNA delivery into photoreceptors has not been reported yet. Here, we investigated the delivery of Cas mRNA together with sgRNA to edit photoreceptors and RPE in adult mice. After subretinal injection of Cas9 mRNA:sgRNA complexed with different lipids, no indels were detected in the neural retina and low percentage of indels were observed in the RPE.

Recent advances in mRNA synthesis allow to decrease its immunogenicity and to enhance its stability (Andries et al. 2015; Devoldere et al. 2016; Henderson et al. 2021; Svitkin et al. 2017). Mature mRNA requires a 5'-cap for gene expression and mRNA stability. We used the cap 1 that showed higher efficiency compared to first-generation cap analogs as ARCA cap (Henderson et al. 2021). Moreover, pseudouridine-5'-triphosphate (Ψ) and 5-methylcytidine-5'-triphosphate (m5C) are used to replace UTP (U) and CTP (C) in the mRNA synthesis, because m5C/ Ψ mRNAs trigger low-to-no immune responses and cytotoxicity into cells (Andries et al. 2015; Svitkin et al. 2017). These optimized chemical modifications already showed to expand applicability of CRISPR-associated gene editing tools *in vitro* and *in vivo* (T. Jiang et al. 2020). However, delivery of large mRNA encoding SpCas9 proteins seems more challenging than reporter proteins. Another parameter is the degradation of sgRNA that is co-delivered with Cas9 mRNA before its complexation with newly expressed Cas9 protein. Here, we choose a ratio mRNA:sgRNA of 1:10 to have an excess of sgRNA. The capacity of retinal cells to translate correctly large mRNA and timeline between mRNA and sgRNA delivery need to be address *in vivo*.

In this study we investigated the potential of non-viral Cas9 mRNA delivery to edit photoreceptors in adult mice. Commercialized lipid-based carriers were evaluated in their capacity to efficiently complex Cas9 mRNA. We tested non-viral vectors commercialized for *in vitro* or *in vivo* RNA delivery. They were able to complex Cas9 mRNA and sgRNA. However, nanoparticles formed were large and polydisperse. After subretinal injection of Cas9 mRNA complexed with Invivolipofectamine 3.0 or invivojetRNA, no indels were detected in the neural retina and low indels were observed in the RPE.

In recent years, non-viral vectors to deliver mRNA-based therapy have been improved and finally reached the clinic followed by FDA approval for several applications ((Administration ; Administration. ; Administration.) To summarize, the first category of non-viral vector developed for gene replacement therapy was cationic liposomes but they are still in the clinical investigation stage. In recent decade, LNP outperformed liposomes to deliver mRNA and received recent approval for LNP-based therapy in the liver (Onpattro®) or mRNA vaccines for COVID-19. Compared to liposomes, LNP do not have a lipid bilayer and a large inner aqueous pool. These two non-viral vectors are composed of natural phospholipids or their derivatives, cholesterol, cationic or ionizable lipids, and polyethylene glycol (PEG)-lipids. Among these components, cationic lipids are essential to condense and protect the negatively charged mRNA. In the past decade, vectors based on permanently charged lipids as commercialized vectors derived from Lipofectamine 2000 have been replaced by ionizable cationic lipids that are more safe and efficient (Polack et al. 2020)

The first applications of Cas9 mRNA delivery were reported in the liver using ionizable cationic lipids. Indeed, liver is an organ of choice due to a strong liver tropism induced by low-density lipoprotein (LDL) receptors on liver cells after intravenous administration (Böttger et al. 2020).

First *in vivo* gene editing therapy based on Cas9 mRNA delivery was applied to the liver and reached the clinic in 2020 (NCT04601051). Intellia Therapeutics' NTLA-2001 was injected into patients for the treatment of transthyretin amyloidosis (Gillmore et al. 2021). Their LNPs had high encapsulation efficiencies (>95%) and were less than 100 nm. In mice, Cas9 protein was detected 4 hours after mRNA delivery and level of edition reached its maximum at 96 hours (Finn et al. 2018). They are biodegradable avoiding bioaccumulation in the liver and

potential safety risks compared to the stability of DLin-MC3-DMA, a lipid currently being used in clinical trials (Mui et al. 2013). In addition, LNPs used in this study were well tolerated and allow a rapid clearance of Cas9 mRNA and sgRNA from the animals, both undetectable after 72 hours in mice (Finn et al. 2018). This most advanced study gave some insights to successfully apply Cas9 mRNA delivery in the retina.

To conclude, transient Cas9 mRNA delivery efficacy is still weak compared to AAV or proteins delivery *in vivo* and more work is needed to identify optimal characteristics of non-viral vector to deliver efficiently mRNA in the retina.

Material and Methods

SpCas9 mRNA and sgRNA synthesis

5moU CleanCap SpCas9 mRNA that contains two NLS was purchased from TriLink. The *VEGFa* sgRNA (sequence: CTCCTGGAAGATGTCCACCA) targeting a common sequence between species of the murine vascular endothelial growth factor A (*VEGFa*) gene was published by Kim and colleagues (Kim et al., 2017). sgRNA were synthesized and purified using the GeneArt™ Precision gRNA Synthesis Kit (Invitrogen), according to the manufacturer's protocol. sgRNA eluted in water were aliquoted and stored at -80°C.

Cas9 mRNA and sgRNA complexation with non-viral vectors

Synthetic vectors were mixed with Cas9 mRNA and sgRNA (ratio 1:10), vortex for 10 seconds and incubated at room temperature 10min for characterization or until injection. Lipofectamine™ 2000 and Lipofectamine™ RNAiMAX Transfection Reagent, InvivoFectamine 3.0™ and MessengerMax™ were purchased from ThermoFischer. InvivojetRNA and jetMessenger were purchased from Polypus.

Dynamic Light Scattering

The hydrodynamic diameter (size) of each complex was measured using DLS (Zetasizer nano analyser ZS; Malvern Instruments) at Paris ESPCI facility. Three different replicates were carried out with 5 minutes in between each measure.

Electronic microscopy

Samples were prepared and visualized by electronic microscopy at Faculté de Pharmacie de Paris. Briefly, samples were placed on carbon-coated grids and negatively stained with 2% uranyl acetate for 4 minutes.

Retardation gel assay for Cas9 mRNA complexation

The degree of complexation of sgRNA, Cas9 mRNA and non-viral vectors listed above was measured by developing a gel retardation assay using a non-denaturing agarose gel. sgRNA (1 µL, 5 µM) and Cas9 mRNA (1 µL, 0.5 µM) were mixed together and incubated for 5 minutes at room temperature, followed by the addition of non-viral vectors with increasing

volume of vectors. A total of 3 uL of complexation solution were formulated and were incubated for 10 minutes at room temperature. Then 3.5 uL of 40% glycerol was added in addition to H₂O to have a final volume of 10.5uL. Solutions were run on a 1.5% agarose gel at 170 V for 20 minutes. For the different control samples (sgRNA alone, SpCas9 mRNA alone and sgRNA:mRNA), reagents were replaced with the RNase-free water. Invivofectamine 3.0™ and InvivojetRNA were diluted in specific buffer provided by the manufacturer.

Subretinal injections in mice

For animals, the experiments were realized in accordance with the NIH Guide for Care and Use of Laboratory Animals (National Academies Press, 2011). The protocols were approved by the Local Animal Ethics Committees and conducted in accordance with Directive 2010/63/EU of the European Parliament.

C57BL/6j wild type (Janvier Labs) mice were used for this study. For ocular injections, mice were anesthetized by isoflurane inhalation. Pupils were dilated and sub-retinal injections of 2 uL were performed using a Nanofil syringe with a 28G blunt needle (World Precision Instruments, Inc.) under an operating microscope (Leica Microsystems, Ltd.). Ophthalmic ointment (Fradexam) was applied after surgery. Eyes with extensive subretinal hemorrhage were excluded from the analysis. Animals were euthanized by CO₂ inhalation and cervical dislocation. After 7 days, the whole RPE and the whole retina were isolated without selection of transfected cells.

Samples preparation for Indels quantification

For genomic DNA extraction, samples from each experimental condition were incubated in lysis buffer and proteinase K at 56°C overnight for retina samples, 3 hours for RPE tissue and 10min for *in vitro* cells. Further steps were performed following the manufacturer's instructions (NucleoSpin® DNA tissue, MACHEREY-NAGEL). We used the GXL HiFi DNA polymerase (Takara) for PCR amplification. Primers used are the following: Forward 5'-CAAATCTGGGTGGCGATAGA-3' and Reverse 5'-AGATGGTCAAATCGTGGAGAG-3'. The thermal cycler program for PCR was as follows: 98° C for 10s, followed by 60° C for 15 s and finally 68° C for 20s, with in total 30 cycles. PCR samples were then purified using following the manufacturer's instructions (NucleoSpin® PCR and gel kit, MACHEREY-NAGEL). Purified amplicons were verified by electrophoresis on 1% agarose gels.

For *in vivo* and *ex vivo* experiments, PCR amplicons were sent to next generation sequencing (NGS) at Massachusetts General Hospital DNA core facility. A total of 10 000 reads were generated per samples and analysis were done with a cut off of 10 reads.

Statistical analysis

All statistical analyses were carried out using GraphPad PRISM version 7.0. Error bars on the graphs show values $\pm$ SD. P-values were determined by non-parametric student t-test.

Discussion and Perspectives

During my PhD thesis, I investigated the feasibility of transient delivery of gene editing tools *in vivo* in the retina in order to treat retinal diseases. As a first proof of concept, I investigated the direct delivery of either Cas9 mRNA or Cas9 RNP in the retina of adult mice. On the contrary to Cas9 mRNA, delivery of Cas9 protein and its sgRNA as ribonucleoprotein (RNP) complex showed a high editing efficiency in the retina, even in absence of any vectors. In this 'vector-free' condition, I observed some early toxicity due to the high concentration of Cas9 protein in the subretinal space. As part of this work, I identified physical barriers specific of the retinal tissue that impede Cas9 protein delivery in mice and in primate retinal tissue and attempted to circumvent these barriers by using non-viral vectors to complex Cas9 RNP and mRNA along with its sgRNAs. The delivery challenges were particularly significant with mRNA suggesting there were additional hurdles in this delivery strategy. These difficulties may include a time lag between Cas9 mRNA translation and preservation of the guides during this process. Although some improvement could be obtained in the RPE delivery using synthetic vectors, most challenges remained for efficient delivery and gene editing in photoreceptors. I learned from this work that synthetic vectors could aid in increasing indel rates in the RPE but not in photoreceptors. This made me ask if other factors such as the efficacy of the guides and the different accessibility of the target locus could play a role, downstream of intracellular delivery. Indeed, as an example target we had chosen the VEGF gene which is not a gene expressed in photoreceptors. By changing from this locus to a gene highly expressed gene in photoreceptors we could indeed increase the indel rates by seven-fold pointing to the importance of post-RNP entry steps in achieving therapeutic gene editing rates. Altogether, this work paves the way for transient delivery of Cas9 and its sgRNA to apply gene editing tools efficiently and safely to the retina. These are discussed in detail below.

Transient delivery of Cas9 and its sgRNA as RNP to retinal cells

In our study, direct sub-retinal delivery of Cas9 protein with 2 NLS signals coupled to sgRNA targeting VEGFa gene was able to generate indels in the RPE cells and photoreceptors after sub-retinal injection in a dose-dependent manner. By targeting SAG gene, we were able to reach 7% of indels in the neural retina of adult WT mice leading to a high decrease of SAG mRNA expression level. Editing efficiency necessary to have therapeutic outcomes needs to be adapted for each disease and strategy. To compare with the most advanced study in the field, Maeder and colleagues estimated at 10% the editing efficacy needed to obtain benefit in the vision of patients affected with LCA10 (Maeder et al. 2019). In this study, AAV-SaCas9 under the photoreceptor-specific promoter GRK1 was delivered subretinally in mice and lead to 44% of productive editing among the transduced cells 6 weeks after injection (1.10^{12} vg/mL). In non-human primate retinal explants, an efficiency of 28% of productive editing was reached among photoreceptors with the same dose. In human retinal explants, they attained an average of 17% of productive editing in photoreceptors.

In our study, sub-retinal injections allow our complexes to be in close contact with the RPE cells and photoreceptors. By analysing separately the RPE cells and the rest of the retina that is composed of 80% of photoreceptors we observed the different behaviour of RNPs in different cells. Thus, our results give a precise idea of edition that can be achieved in the whole retina taking into account different obstacles to overcome for different cell types.

In comparison, Maeder and colleagues determined that 30% of mouse retina is transduced by their AAV and calculated their productive editing rate among these cells (Maeder et al. 2019). Previous studies showed Cas9 RNP delivered with non-viral vectors to the retinal pigment epithelium (RPE), resulted in 22% indels within the transfected cells (K. Kim et al. 2017b) and a more modest indel rate of 6% was achieved in the VEGFa locus in isolated EGFP positive RPE cells (A. B. Holmgaard et al. 2021). Isolation of transfected cells can give insight on how many cells are transfected and the editing efficiency among them. However, we did not succeed in isolating transfected cells by cell sorting after injection of Cas9 protein

fused to GFP. Our results are thus difficult to compare to previous work done in the RPE cells but point to a good efficacy without any vector.

Percentage of indels *in vitro* in a murine cone cell line are higher compared to those found *in vivo* but *in vitro* experiments do not always predict the percentage of indels that we can achieved *in vivo*. Indeed, sgRNA targeting *VEGFa* and *SAG* genes have similar indel rate *in vitro*, however *in vivo* differences between these two targets reflects the necessity to optimize the delivery method in a model that represents the genomic landscape of the target cell. Note that we tested Cas9 RNP delivery complexed with cationic lipids in retinal organoids. However, we did not observe any indels after transfection or electroporation of RNPs into 3D organoids at different stages of maturation. More work is needed to optimize RNP delivery in this specific model. 2D Culture of induced pluripotent stem cell (iPSc) derived photoreceptors can facilitate the transfection *in vitro*.

In our study, we show the high potential of Cas9 RNP delivery to edit efficiently the photoreceptors *in vivo*. To achieve therapeutic gene edition, we investigate potential obstacles that prevent RNP delivery in the retina and try to bypass them with non-viral vectors. Our findings are discussed in the following paragraphs.

What are physical barriers that prevent Cas9 delivery into retinal cells?

The neural retina is composed of three compact layers of cells embedded in a tight extracellular matrix (Thorne and Nicholson 2006). Indeed, external segments represent 2/3 of length of rods and are first barriers encountered after subretinal delivery. Volume of extracellular matrix surrounding the photoreceptors decreases from external segments to PRs' cell bodies (Kuo et al. 2020). In addition, an outer limiting membrane (OLM) is present just above the cone cell bodies. Altogether, these barriers prevent diffusion of Cas9 RNP complexes into the photoreceptor layer. Indeed, we show that 3 days after RNP injection, Cas9 protein aggregates are still present on the outer segments and very little Cas9 is visible in and around the cell bodies of photoreceptors. These barriers are common to other species as we observed in non-human primate retinal explants.

They can explain in part the high Cas9 RNPs concentration needed in the subretinal space to obtain edition in the neural retina. In contrary to dense neural retina tissue, RPE consists of a single layer of cells with a high phagocytosis rate. These differences can explain the higher rate of indels that we found in these cells.

In the context of retinal degeneration, structure of extracellular matrix and the limiting membranes of the retina are disrupted (Al-Ubaidi et al. 2013; Kolstad et al. 2010). We hypothesized that RNP delivery in a retinitis pigmentosa mouse model at a stage after the degeneration of external segments may help bypass this physical barrier and increase RNP delivery and in consequence increase the indel rates. Surprisingly, we did not see improvement in indel rates in this specific context. It is likely that the access to the photoreceptor layer was improved but the failure to generate indels could be an additional obstacle in degenerating cells. It is known that, the response to DNA damage in the nervous system occurs in a distinct spatiotemporal manner utilizing different DNA repair pathways to ensure genomic stability and to prevent disease and that these DNA repair mechanisms are compromised during neurodegeneration (Katyal and McKinnon 2007). Degeneration of the neural retina therefore likely triggers other limitations for genome editing that are discussed below.

To stay in a physiological context, a future step is to test our RNP delivery in neonatal mice. Compared to mature retina, early postnatal retina has some advantages with a less compact photoreceptor layer with larger extracellular matrix space between cells and this might present a better opportunity to tease apart the role of extracellular matrix from neurodegenerative mechanisms that occur during RP (Kuo, Chiang et al. 2020).

What are the potential causes of microglial activation after RNP delivery into the subretinal space?

One month after Cas9 RNP injection, we observed activation of microglial cells in retinal flatmounts. This result is coherent with OCT data that showed a decrease of the ONL layer in some of injected eyes compared to control eyes. Cas9 RNPs can trigger toxic signals and potential activation of immune responses in the retina. Cross section of retina will allow us to define in which layer activated microglia cells are present. These early immune responses

can be explained by the high concentration of Cas9 proteins in the subretinal space with potential aggregation as well as the foreign origin of our SpCas9 protein. Indeed, a previous study reported signs of toxicity at high Cas9/gRNA concentrations (A. B. Holmgaard et al. 2021).

At a lower level, we observed activation of microglia cells in control eyes compared to non-injected eyes. Control eyes were injected with Cas9 RNP suspension buffer (composed of 20mM of HEPES and 200mM of KCl). Signs of toxicity in controls can be explained by the high concentration of KCl in this solution compared to physiological composition of extracellular space.

Our volume of injection of 2uL is also a concern and can explain the activation of microglial cells as previously shown in the lab (unpublished observations). As we do not see significant differences of edition efficiency between 1uL and 2uL injection, we could decrease our volume of injection in future experiments. Another possibility is to complex RNPs with non-viral vectors to protect them from degradation, improve their cellular entry and shield them from immune system. This would require that the vectors used do not elicit immune reactions by themselves.

Which type of non-viral vectors to deliver Cas9 RNP to the retina?

Complexation of the RNPs with non-viral vectors can bypass physical barriers mentioned above and improve intracellular delivery. To this aim, we tested cationic lipids that showed a capacity to complex and deliver RNPs into RPE cells and inner ear cells (Gao et al. 2018; A. Holmgaard et al. 2017; A. B. Holmgaard et al. 2021; K. Kim et al. 2017b; Zuris et al. 2015). Indeed using cationic lipids, we see slight improvement of the edition in the RPE but not in the neural retina. Contrary to RNAiMAX, Lipofectamine 2000 provides indel rate comparable to RNPs alone in the retina.

RNPs observed in DLS are small and homogenous complexes of 37.5 nm which is coherent with previous studies (Nguyen et al. 2020). In contrary, RNP complexation with cationic lipids leads to polydisperse and big complexes. The size of RNP complexes and their capacity to diffuse are important parameters to develop RNP delivery in neural retina. On the contrary, larger complexes formed with cationic lipids did not impact delivery into RPE cells.

We can note the high variability between samples for this condition that can be explained by the polydispersity and the low stability of complexes observed in DLS. Additional eyes are needed for this condition to conclude on the potential of Lipofectamine 2000. In addition, as sgRNA targeting SAG gene leads to higher indel rate, comparison between RNP delivered alone or complexed with Lipofectamine 2000 with this sgRNA will be easier.

Finally, Lipofectamine 2000 is known to be toxic (Zuris et al. 2015) and may prevent its use to deliver RNP complexes *in vivo*.

As an alternative to lipids, we tested other non-viral vectors for their capacity to complex RNPs, form small complexes and improve intracellular delivery. SP10 peptide is a small 34 amino acid peptide composed of a cell penetrating peptide and an endosomolytic peptide that has been reported to enhance intracellular delivery in cultured human ciliated and non-ciliated epithelial cells and mouse airway epithelia (Krishnamurthy et al. 2019). In our hands, Cas9 RNPs complexed with SP10 form heterogeneous complexes ranging from 34.4 ± 3.7 nm to large complexes of 2766 ± 288 nm. First peak of 34.4 nm can correspond to Cas9 RNP alone that suggests only partial complexation of RNP with SP10 lack of improvement in indels in the neural retina.

However, peptides covalently linked to RNP could improve intracellular delivery as previously shown in the brain with Cas9 protein fused to multiple NLS sequences (Staahl et al. 2017). In this study, additional NLS are using as cell penetrating peptides to improve intracellular delivery. Interestingly, same dose dependant RNP concentration was used to edit post-mitotic neurones (Staahl et al. 2017). Covalent link will ensure formation of smaller and more homogenous complexes. Currently, we are testing Cas9 protein with 6NLS and we wish to compare them to Cas9 with 2 NLS *in vivo*. Other peptide modifications as SP10 peptide could improve complexation, intracellular delivery efficiency, endosomal escape and targeting specificity (Chen et al. 2019; Krishnamurthy et al. 2019).

In conclusion, non-viral vectors are of value for RNP complexation in order to decrease the Cas9 concentration needed and thus potential toxic signals associated. It is not yet clear if cationic lipid as Lipofectamine 2000 or covalent multiple NLS will improve edition in photoreceptors. Moreover, new vectors are in constant development to complex Cas9 RNP

into small nanoparticles that allow efficient edition in various cell type (Banskota et al. 2022; Zou et al. 2022).

We can note a new category of vectors named nanoblade (also referred to as Virus-Like Particles (VLPs)) that are based on viral vectors that deliver RNP instead of DNA (Mangeot et al. 2019). However, this vector derived from enveloped viral vector murine leukemia virus is too large to efficiently transfect photoreceptors (Banskota et al. 2022).

How will these guiding principles of delivery be applicable for other editors?

Our study focuses on the delivery of Cas9 protein with sgRNAs. Today, this tool is already used in clinic and allows to knockout a gene or to remove a specific region (Maeder et al. 2019). Next step is to deliver base and prime editors that extend the promise of gene editing approaches to correct 99.9% of IRD. As these new tools don't fit into one AAV, transient delivery methods are necessary but remain challenging. dCas9 nuclease is coupled with a deaminase (CBE and ABE) or an engineered reverse transcriptase enzyme (prime editing). First limitations are the production and the purification of these proteins. Then, the molecular weight of base and prime editors, respectively 200kDa and 220kDa, will change optimal parameters set up for SpCas9 protein (160kDa). In addition, prime editors must be delivered in each cell with one sgRNA and one pegRNA. Delivery of multiple compounds of different nature is challenging and a stable vector is needed to deliver all compounds with the best ratio in each cell.

Transient delivery of Cas9 as mRNA and its sgRNA in the retina

A first clinical based on the liver delivery of SpCas9 mRNA using lipid nanoparticle (LNP) based vectors paves the way to develop Cas9 mRNA base therapy (Gillmore et al. 2021). Reporter gene mRNA delivery in the retina has been reported in recent studies (Devoldere et al. 2019; Patel et al. 2019; Ryals et al. 2020). Proof of concept of mRNA encoding GFP protein has been shown in photoreceptors of wild-type mice (Devoldere et al. 2019). However, delivery of the larger mRNAs encoding Cas9 protein is likely more challenging. During my PhD, I also investigated the delivery of Cas9 mRNA to edit the neural retina and the RPE. First, I tested several commercialized cationic lipids for their ability to complex Cas9 mRNA:sgRNA and to form small complexes. Two commercialized vectors specific for *in vivo* applications were selected for our *in vivo* study in mice. After subretinal injection of Cas9 mRNA complexed with these two lipids, no indels were detected in the neural retina and only few indels were observed in the RPE. More work is needed to identify optimal characteristics of non-viral vector to deliver efficiently mRNA in the retina. Patel et al have screened several cationic ionizable lipids (CIL) and showed the capacity to complex mRNA with ionizable lipids to form small particles of 80nm that lead to high protein expression in the RPE (Patel et al. 2019). In addition, the capacity of retinal cells to translate correctly large mRNA and time lag between mRNA and sgRNA delivery have not been investigated in this study.

mRNA mediated expression is an attractive strategy for delivery of gene editing tools and offer a modular alternative for base editors or prime editing tools that do not fit into a single AAV and are not easy to produce as proteins.

In vivo gene editing in the retina

Repair efficiency in the retina

Is *in vitro* validation of sgRNA relevant for *in vivo* applications?

Our results raised some questions about repair efficiency in the retina. First, sgRNA targeting VEGFa gene induced similar indel rates in a murine cone cell line compared to sgRNAs targeting highly expressed SAG gene. These results were not coherent with *in vivo* studies where sgRNA targeting SAG gene induced 10 times more edition compared to sgRNA targeting VEGFa gene. We can note that indel profile found in these two models were the same for each sgRNA that suggest same repair mechanisms are involved in these two models but with a different efficiency rate. Chromatin accessibility to Cas9 protein is a key parameter for editing efficiency. It has been shown that genes in heterochromatin are less accessible for Cas9 than genes in euchromatin (Wu et al. 2014). Indeed, VEGFa gene is not expressed in the neural retina rather than SAG gene which is highly expressed in photoreceptors. Moreover, gene expression and thus DNA accessibility are different between a mitotic cell line and *in vivo* post-mitotic photoreceptor cells and these can partially explain our low efficacy results.

Importantly, our results question the accuracy of *in vitro* sgRNA validation. Direct validation of sgRNA *in vivo* could be applied by developing a high-throughput screening method in the retina. For example, we could screen multiple sgRNA delivered by an AAV with a mouse strain expressing Cas9 (M. Kuhn 2021).

We observed an increase of indel rate one month after injection compared to 1 week. Eye fundus imaging showed that majority of Cas9 fused to GFP is no longer present in the subretinal space at D7. One hypothesis to explain the delay between the delivery of Cas9 protein and formation of indels is the low accessibility of VEGFa gene in the retina *in vivo*. By targeting gene highly expressed in the photoreceptors such as the SAG gene, we could improve the accessibility for repair proteins and do not observe the same kinetics of reparation as we did for the VEGFa gene. Indel rates of Cas9 RNPs targeting SAG gene at 1

month will allow us to confirm this hypothesis. Impact of DNA accessibility on Cas9 cleavage efficiency could lead to the use of local chromatin modulation to enhance indel rates (Chung et al. 2020).

In addition to DNA accessibility to Cas9 system, editing efficiency seems to be dependent on double stranded break (DSB) repair efficiency. Repair mechanisms and its efficiency in the retina are not well described. Only one study investigated efficiency of DSB repair pathway inside the retina after irradiation of mice *in vivo* or retinal explants of different species. Inside a particular model, editing efficiency seems to be dependent on the age and cell type (Frohns et al. 2020). In mice, repair of DSB is more efficient in cones compared to rods (Frohns et al. 2020).

Our results in rd10 mouse model of retinitis pigmentosa raise questions about the DSB repair efficiency *in vivo* in the context of degenerative retina. The lower editing efficiency found in rd10 retina compared to WT retina could be explain by repair defect due to cell degeneration as previously shown (Al-Ubaidi et al. 2013). Stage of the degeneration will have a big impact on the efficiency of gene editing. On the contrary, photoreceptors of mice pups seem to have a higher capacity to repair their DNA (Frohns et al. 2020).

In conclusion, after a successful delivery of gene editing tools, gene editing efficiency at the DNA level are not well understood in the retina and more work is needed. In addition, the therapeutic window needs to be addressed for each disease and take into account potential decrease of the repair mechanism.

Finally, each gene editing tool triggers different repair pathways inside the cells. Efficiency of these pathways needs to be better understood in the particular context of the retina.

Cas9 RNP delivery in Usher syndrome

Usher syndrome type 1 B (USH1B) is due to mutations on the MYO7a gene. Long coding sequence of this gene prevents it from being corrected with a gene replacement therapy. In this context, gene editors are a promising approach to correct Usher syndrome (Fry et al. 2021). Our long-term goal is to apply our transient RNP delivery in the retina and the inner ear to correct precisely mutations leading to USH1B in ciliated cells *in vivo*.

During my PhD, I set up iPSC culture of a USH1B patient that presents heterocompound mutations with 1 substitution (c.3719G>A) and one deletion of 3 pb (c.5886-5888del). Both mutations can be corrected by prime editing. In addition, shaker mice and humanized mice for *MYO7a* gene are available at the Institut de l'Audition to test our strategy *in vivo*. In a long-term perspective, I changed the background of shaker mice from BALB/c to B6J that will allow following of the melanosomes in RPE cells more easily as a phenotypic readout for efficient gene correction. Indeed, shaker mice do not present retinal degeneration phenotype that one can record with functional measures.

To test RNP delivery in the inner ear, our strategy is to perform injection of 1uL of Cas9 RNPs through the round window membrane in WT mice at P1-P2. This injection allows close contact with hair cells. We will analyse separately the supporting cells from the sensory cells. This work is done in collaboration with Aziz El-Amraoui group in Institut de l'Audition.

Transient Cas9 protein delivery has already been successfully applied in the inner ear (Gao et al. 2018; Zuris et al. 2015). Thanks to our study in the retina, we hypothesized that inner ear protocol presents several advantages for Cas9 RNP delivery compared to retina tissue. First, hair cells form a layer of accessible cells in the inner ear that are surrounded by the endolymph. In addition, neonatal animal could present a more permissive both in view of physical barriers and higher capacity for DNA repair.

In conclusion, this work showed the challenges to deliver efficiently Cas9 as a protein or mRNA into photoreceptor cells *in vivo*. First, Cas9 needs to bypass specific extracellular barriers as the outer segments of the photoreceptors, then to enter efficiently into the cells and escape the endosomes. One concern to be addressed is the microglial activation due to the high RNP concentration in sub-retinal space. This work opens the way to deliver transiently gene editing tools into the retina and in other neural tissues as the inner ear and the brain. Finally, as a more global contribution to the field, we highlight the necessity to investigate the efficiency of gene editing and repair mechanisms in a specific tissue in a specific model with the adequate genomic landscape such as chromatin accessibility.

Annexes

Annex 1: USH1B iPS cell model

In a previous study, induced pluripotent stem cells (iPSCs) were generated from a patient with compound heterozygous MYO7A mutations (c.1184G>A and c.4118C>T), and one of the MYO7A mutation sites (c.4118C>T) was corrected using the CRISPR-Cas9 system, thus showing the feasibility of this approach at iPSC stage (Tang et al. 2016). It was further demonstrated that the corrected iPSCs retained their cellular pluripotency as well as normal karyotypes and were used to obtain hair cells with improved structure and physiology.

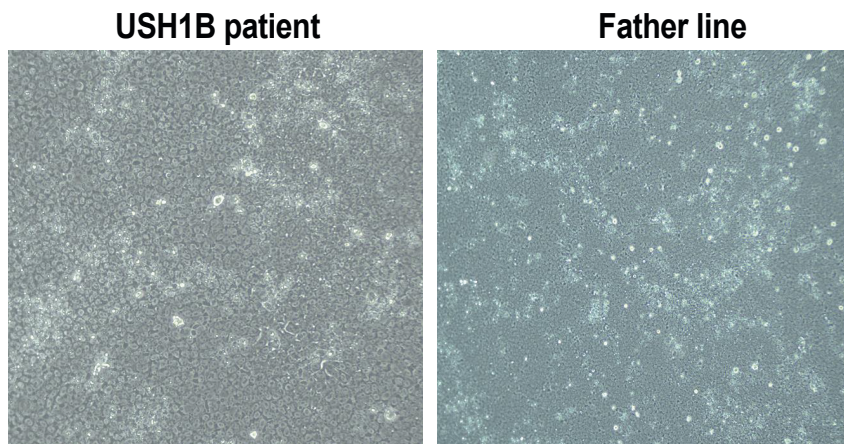
During my PhD, I set up an iPS cell model in order to obtain 3D retinal organoids of Usher syndrome type 1 B (USH1B). Characterization of these organoids will provide a better understanding of USH1B disease as mice models do not present any retinal phenotype (El-Amraoui and Petit 2014). Moreover, it will allow us to test our gene editing strategy in a human genomic context, in addition to our *in vivo* work in mouse models.

Among genotyped French USH1B patients, we selected one patient with heterozygous mutations: a substitution c.3719G>A (p. Arg1240Gln) and a 3pb deletion c.5886_5888del (p. Phe1963del) (Fig 1B). Both of these mutations are recurrent in the population and c.3719G>A is the most frequent mutation in the French and German population. We will attempt a correction thanks to the newly developed prime editor. We decided to first attempt the correction of the substitution (mother's mutation) and thus choose the father cells as control.

First, iPS cells were reprogrammed from human peripheral blood mononuclear cells (PBMC) of patient USH1B and patient's father lines by Phenocell Company. Culture was done in xeno-free, feeder-free conditions. One clone of each line were characterized at passage 10 and showed correct morphology (Fig.1A) and karyotype (Fig.2E). Mycoplasma test was negative. Surface markers of pluripotency were analysed by flow cytometry and are above 85% of total cells (Fig.2A). Pluripotency was also confirmed by immunostaining of OCT4, SOX2 AND NANOG markers (Fig.2B). Finally, differentiation potential by embryoid body (EB) formation showed the presence of cells from the 3 germ layers (Fig.2C).

We differentiated these two cell lines into pseudolaminated retinal organoids that contained all major retinal cell types thanks to a protocol published by Olivier Goureau's team at Vision Institut (Fig. 3A; (Slembrouck-Brec et al. 2019). Patient iPSC's retinal organoids showed same morphology compared to control ones at early stage of development (Fig. 3B). Two clones of each lines were tested, however, only few retinal organoids were obtained preventing a full characterization of USH1B retinal organoids. This project is continuing now in Institut de l'Audition.

A.



B.

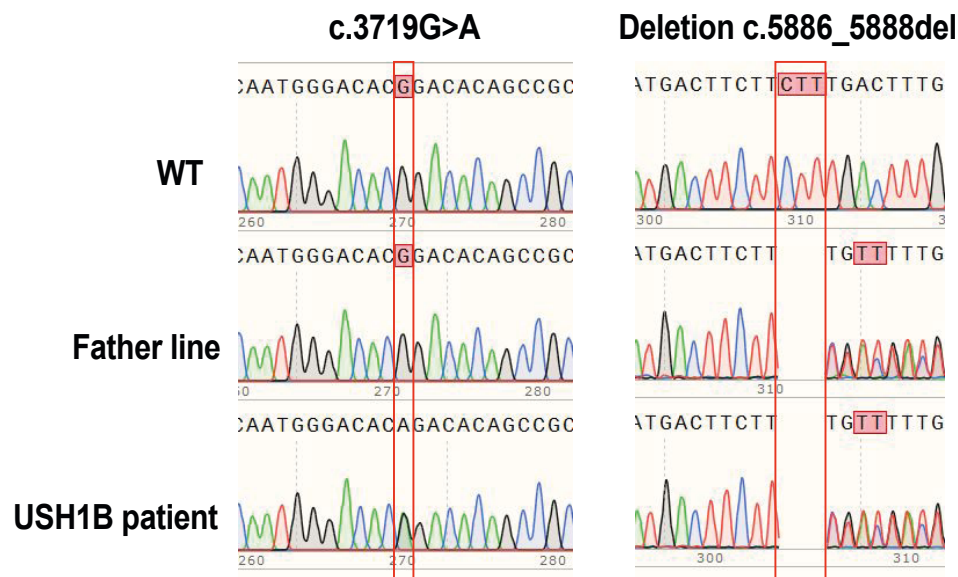
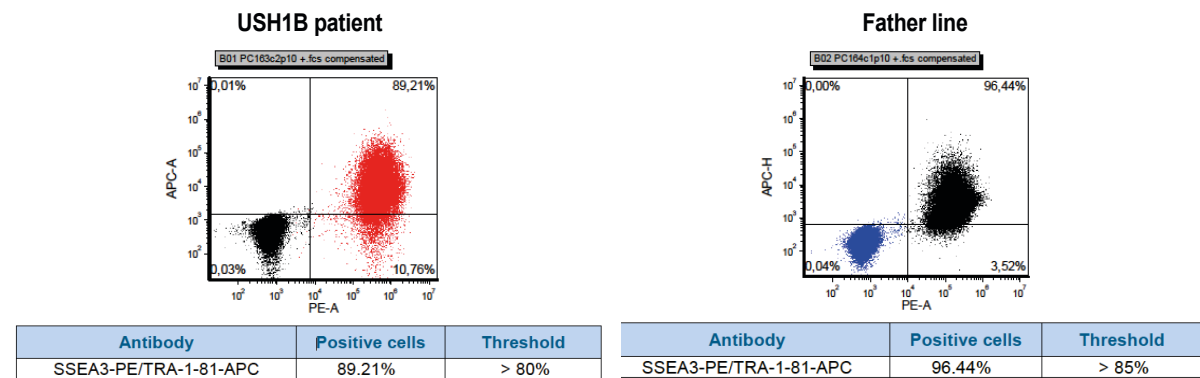


Figure 1: USH1B iPS cells and its control.

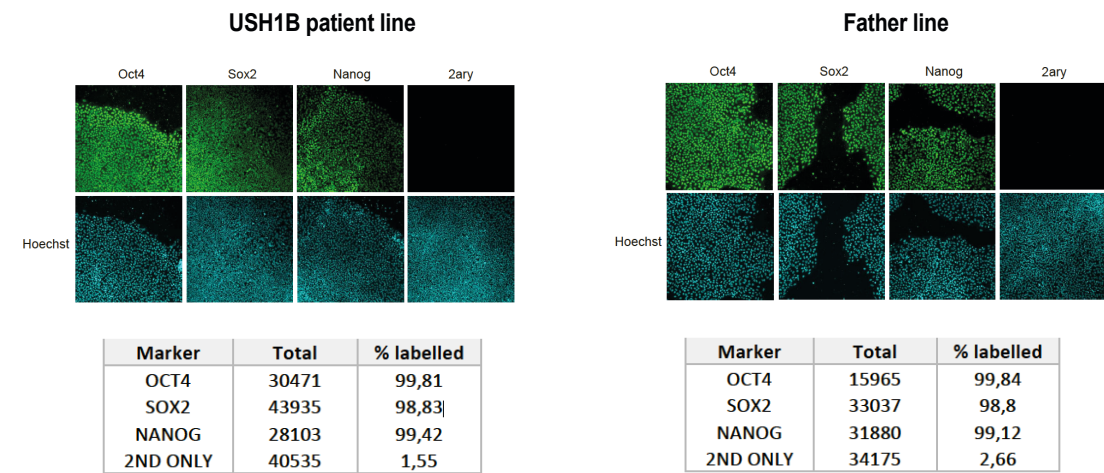
A. Morphology of iPS cell line derived from USH1B patient (PC164 clone 1 at passage 10) and father's patient (PC163 clone 2 at passage 10). IPS cells have been cultivated in feeder-free, xeno-free, mTeSR1 (Stemcell technologies #85850).

B. Sanger sequencing of the two mutations found in USH1B patient line compared to normal cells and cells from the patient's father.

A.



B.



C.

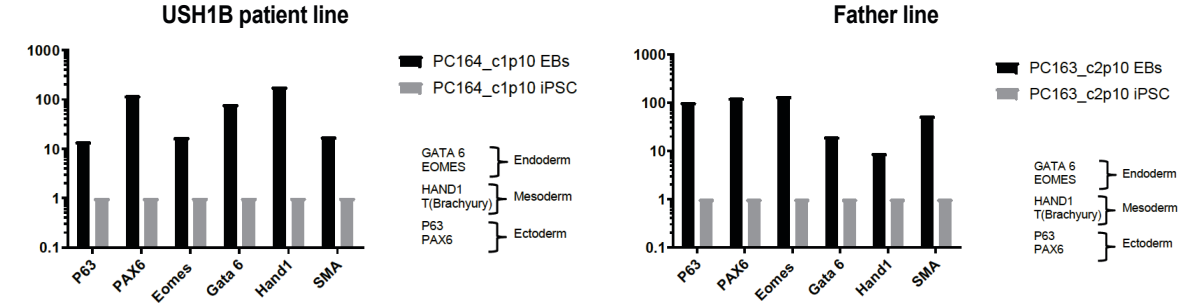
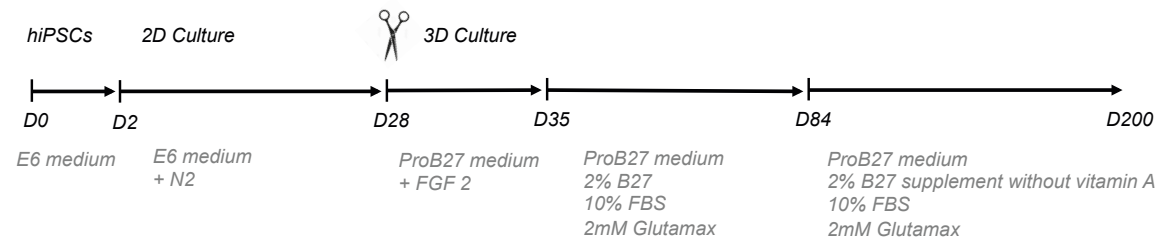


Figure 2: Characterization of iPSC at passage 10.

- A. Analysis of surface marker of pluripotency by flow cytometry.
- B. Immunostaining of OCT4, SOX2 and NANOG markers. Labelled cells were quantified with CellInsight™ CX5 imager (one 24-well plate have a surface of 1.8 cm²).
- C. EB formation and presence of cells from the 3 germ layers by RT-qPCR in USH1B patient line (PC164 clone 2) and father's patient line (PC163 clone 2).
- D. R-banding karyotyping (RHG, resolution 400-500 bands), 10 metaphasis.

A.



B.

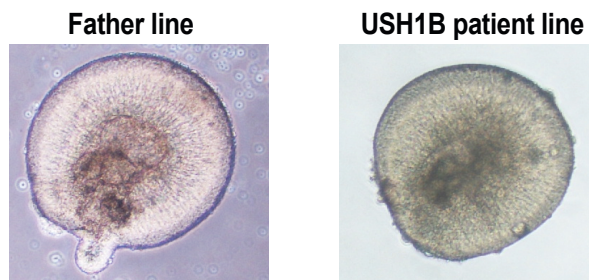


Figure 3: Retinal organoids derived from USH1B iPSC cells.

A. Protocol of differentiation used to obtained pseudolaminated retinal organoids.

B. Retinal organoids (day 35) derived from father and USH1B patient iPSC line.

Annex 2: Poster for ASGCT congress



Transient CRISPR-Cas delivery for therapeutic gene editing in the retina

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How to deliver Cas9:sgRNA into photoreceptors ?

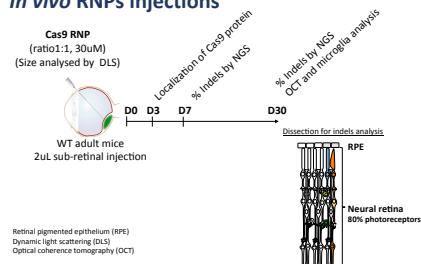
Cas9 protein
160 kDa
Charge +22

sgRNA
Charge -101

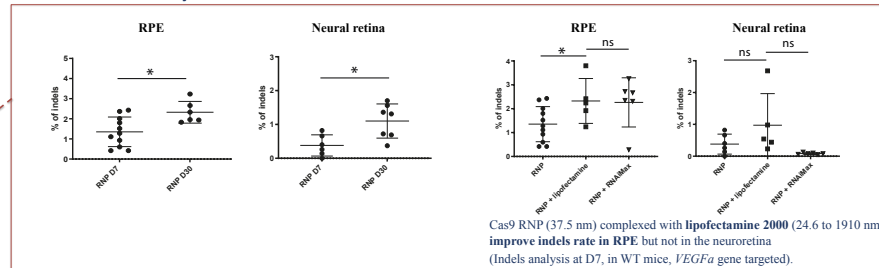
Ribonucleoprotein complex (Cas9 RNP)

Cas9 RNP delivery has been already reported in the RPE*

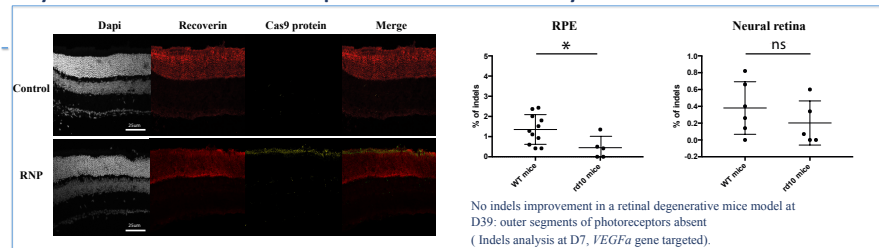
In vivo RNPs injections



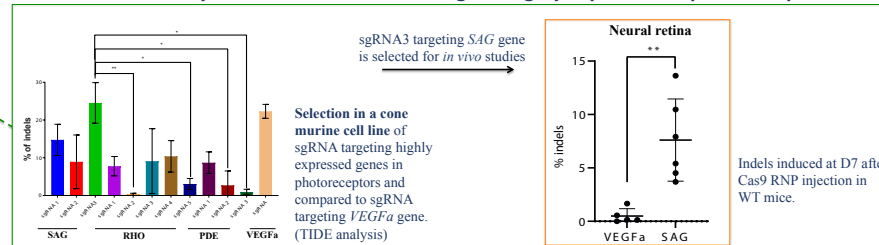
Cas9 RNPs delivery induces indels *in vivo* in the neural retina and in the RPE



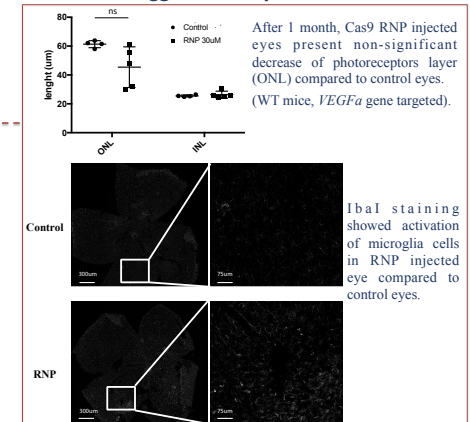
Physical barriers of neural retina prevent Cas9 RNPs delivery *in vivo*



Cas9 RNPs efficiently induces indels *in vivo* in a gene highly expressed in photoreceptors



Cas9 RNPs triggers toxicity inside the retina



Take home messages

Direct delivery of Cas9 RNPs generated indels in the retina *in vivo*.

Cationic lipids were able to complex Cas9 RNPs and increase efficacy towards RPE delivery but they did not increase indels in the neural retina.

Imaging labeled RNP complexes showed accumulation above the tightly stacked outer segments.

Cas9 RNP delivery in mice with degenerated outer segments did not improve indels suggesting other physical cellular barriers are at play.

The gene target and efficacy of the sgRNA are important as illustrated by the several fold increase.

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Electronic microscopy facility at Faculté de Pharmacie de Paris
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Abstract

Genome editing mediated by CRISPR-Cas has shown promise for the treatment of retinal dystrophies (RD). Currently, adeno-associated viruses are the most widely used vectors for retinal gene therapies but their small packaging capacity and permanent transgene expression makes them suboptimal for CRISPR-Cas delivery. Transient delivery of Cas9 protein and its gRNA as ribonucleoprotein (RNP) complexes have been reported in the retinal pigment epithelium (RPE) and into the inner ear cells *in vivo*. We investigate transient delivery of either Cas mRNA or Cas RNP into photoreceptors as these are the target cells for most prevalent inherited retinal degenerations. Cas9 mRNA complexed with different lipids leads to a few indels *in vivo* in the RPE only. On the contrary, subretinal injection of Cas9 RNP generated indels in the neural retina in a dose dependent manner. Targeting VEGFa gene, the indels reach 0.4% in the whole neural retina compared to 1.4% in the RPE. To improve delivery, we investigated different categories of non-viral vectors such as cationic lipids and cell penetrating peptides (CPP). These synthetic carriers successfully complexed Cas RNP and increased its delivery to the RPE but not in the neural retina. RNP complexes showed accumulation above the tightly stacked outer segments pointing to physical barriers to neural retina infiltration. Delivery in mice lacking outer segments did not improve indel frequency suggesting other barriers such as the accessibility of the target gene and DNA repair mechanisms in degenerating photoreceptors are at play. Indeed, changing the target locus from VEGFa to SAG, a gene highly expressed in photoreceptors, improved significantly indel frequencies reaching 7% of indels and leading to SAG mRNA level decrease. Our results highlight the need to develop new carrier compounds and use suitable model systems for validation of gene specific interventions using CRISPR Cas.

Keywords : Gene editing, CRISPR Cas, ribonucleoprotein, retina, retinal dystrophies

Résumé

L'édition du gène par CRISPR-Cas s'est révélée prometteuse pour le traitement des dystrophies rétiniennes (DR). De par leur faible capacité d'insert et l'expression permanente de leur transgène, les vecteurs adéno-associés ne sont plus optimaux pour le transfert de CRISPR-Cas. L'administration transitoire de la protéine Cas9 et son ARNg sous forme de complexes ribonucléoprotéiques (Cas9 RNP) a déjà montré un transfert efficace à l'épithélium pigmentaire rétinien (EPR) et dans les cellules de l'oreille interne *in vivo*. Ici, nous avons étudié le transfert direct de Cas9 sous forme d'ARNm ou de RNP dans les photorécepteurs qui sont les cibles principales dans les DR héréditaires. Le transfert de l'ARNm Cas9 complexé avec différents lipides a entraîné une édition uniquement dans l'EPR *in vivo*. Au contraire, l'injection sous-rétinienne de Cas9 RNP a généré des indels dans la rétine neurale de manière dose-dépendante. Pour améliorer le transfert, nous avons étudié différentes catégories de vecteurs non viraux tels que les lipides cationiques et les peptides. Ces transporteurs synthétiques ont augmenté leur transfert dans l'EPR uniquement. Les complexes RNP ont montré une accumulation au-dessus des segments externes indiquant des barrières physiques empêchant l'infiltration des RNPs dans la rétine. L'administration chez des souris dépourvues de segments externes n'a pas amélioré la fréquence des indels, ce qui suggère que d'autres barrières telles que l'accessibilité du gène et les mécanismes de réparation de l'ADN dans les photorécepteurs en dégénérescence sont en jeu. En effet, le changement du locus ciblé a permis d'améliorer la fréquence des indels de manière significative, atteignant 7% et entraînant une diminution du niveau d'ARNm pour le gène *SAG*. Nos résultats mettent en évidence la nécessité de développer de nouveaux vecteurs et d'utiliser des modèles appropriés pour la validation des thérapies d'édition du génome.

Mots clés: Édition de gène, CRISPR Cas, ribonucléoprotéine, rétine, dystrophies rétiniennes