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RNA-targeting strategies as a platform for ocular gene therapy

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Article Highlights

- RNA-targeted therapeutics expand the gene therapy toolbox.
- Clinical trials promise RNA-targeted therapies for eye disease within the decade.
- Emergence of CRISPR-Cas RNA editing might accelerate gene therapy for blindness.

Abstract

Genetic medicine is offering hope as new therapies are emerging for many previously untreatable diseases. The eye is at the forefront of these advances, as exemplified by the approval of Luxturna® by the United States Food and Drug Administration (US FDA) in 2017 for the treatment of one form of Leber Congenital Amaurosis (LCA), an inherited blindness. Luxturna® was also the first *in vivo* human gene therapy to gain US FDA approval. Numerous gene therapy clinical trials are ongoing for other eye diseases, and novel delivery systems, discovery of new drug targets and emerging technologies are currently driving the field forward. Targeting RNA, in particular, is an attractive therapeutic strategy for genetic disease that may have safety advantages over alternative approaches by avoiding permanent changes in the genome. In this regard, antisense oligonucleotides (ASO) and RNA interference (RNAi) are the currently popular strategies for developing RNA-targeted therapeutics. Enthusiasm has been further fuelled by the emergence of clustered regularly interspersed short palindromic repeats (CRISPR)-CRISPR associated (Cas) systems that allow targeted manipulation of nucleic acids. RNA-targeting CRISPR-Cas systems now provide a novel way to develop RNA-targeted therapeutics and may provide superior efficiency and specificity to existing technologies. In addition, RNA base editing technologies using CRISPR-Cas and other modalities also enable precise alteration of single nucleotides. In this review, we showcase advances made by RNA-targeting systems for ocular disease, discuss applications of ASO and RNAi technologies, highlight emerging CRISPR-Cas systems and consider the implications of RNA-targeting therapeutics in the development of future drugs to treat eye disease.

Keywords: RNA editing, antisense oligonucleotides, RNA interference, CRISPR-Cas13, ocular gene therapy

Contents

Abbreviations

AAV - adeno-associated virus
ACHM - achromatopsia
ADAR - adenosine deaminase acting on RNA
AdRP - autosomal dominant retinitis pigmentosa
Ago2 - Argonaute 2
AMD - age-related macular degeneration
ANXA4 - Annexin 4A
APOBEC - Apolipoprotein B MRNA Editing Enzyme
ASO - antisense oligonucleotide
B4GALNT1 - beta-1,4-N-Acetyl-Galactosaminyltransferase 1
BG - O6-benzyl-guanine
CARMEN - Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids
Cas - CRISPR associated
CIRTS - CRISPR-inspired RNA-targeting system
CMV - cytomegalovirus
CNV - choroidal neovascularisation
COXI/II - cytochrome oxidase subunit I/II
CRISPR - clustered regularly interspaced short palindromic repeats
crRNA - CRISPR RNA
CRUIS - CRISPR-based RNA-United Interacting System
CURE - C-to-U RNA editor
DD - deaminase domain
DR - diabetic retinopathy
FDA - Food and Drug Administration
EMA - European Medicines Agency
HEPN - higher eukaryotes and prokaryotes nucleotide-binding
ICAM-1 - intercellular adhesion molecule I
IGF-I - insulin growth factor I
IND - Investigational New Drug
IRD - inherited retinal degeneration
LCA - Leber Congenital Amaurosis
lncRNA - long non-coding RNA
m^6A - N^6-Methyladenosine
MCP - MS2 bacteriophage coat protein
METTL3/14 - methyltransferase like 3/14
miRNA - microRNA
mRNA - messenger RNA
ncRNA- non-coding RNA
NMDA - N-methyl-D-aspartate
NUC - nuclease
OTC - ornithine transcarbamylase
P23H - proline to histidine knock-in mutation at position 23
PAC-MAN - prophylactic antiviral CRISPR in human cells
PAM - protospacer adjacent motif
PAMmers - PAM presenting oligonucleotides
PEG - polyethylene glycol
PFS - protospacer flanking sequence
piRNA - piwi-interacting RNAs
PLL - poly-L-lysine

PNA - peptide nucleic acid
Ptbp1 - polypyrimidine tract-binding protein 1
RCas9 - RNA-targeting Cas9
REPAIR - RNA editing for programmable A to I replacement
RESCUE - RNA editing for specific C-to-U exchange
RFP - red fluorescent protein
RGC - retinal ganglion cell
RHO - rhodopsin
RNAi - RNA interference
RP - retinitis pigmentosa
rRNA - ribosomal RNA
RSV - respiratory syncytial virus
scAAV - self-complementary AAV
siRNA - small interfering RNA
SNVs - single nucleotide variants
tRNA - transfer RNA
VEGF - vascular endothelial growth factor
VEGFR - vascular endothelial growth factor receptor-1

1 Introduction

Genetic medicine (or gene therapy) refers to the therapeutic use or manipulation of genes and their expression to ameliorate or cure genetic disease. The development of drugs that target genetic diseases has long been central focus of scientific research. Particularly, the eye has been a leading organ for the development of gene therapies due to being physically separated, easy to access, immune-privileged, and postmitotic. The small, compartmentalised structure of the eye also means there is limited spread to other organs and low dosages can be sufficient for therapeutic benefit. In addition, several non-invasive techniques such as optical coherence tomography (OCT), adaptive optics imaging, microperimetry and electroretinogram (ERG), are available to study structure and function.

Ocular gene therapy began with the seminal antisense oligonucleotide (ASO) therapeutic fomivirsen for cytomegalovirus (CMV) retinitis and has progressed to the recent FDA approval of the first ocular gene therapy voretigene neparvovec-rzyl (Luxturna®) for one form of Leber's Congenital Amaurosis (LCA), delivered in adeno-associated virus (AAV). AAVs represent another significant advance for gene therapy and remain the vector of choice for therapeutic development due to their safety profile and transduction capabilities. Emerging delivery technologies such as lipid nanoparticles are also notable and now expanding the scope of gene delivery to the eye.

In recent times, RNA has garnered much public attention. Besides an eventful history revealing a multitude of functions (**Figure 1**), the molecule has become particularly known for its unstable and transient nature. RNA-targeted therapy is therefore proving to be an attractive alternative to traditional genomic therapies, and providing unique opportunities and challenges for therapeutic development (Damase et al., 2021).

In drug development, RNA-targeted strategies are gaining traction for allowing specific and reversible genetic manipulation that is independent of DNA. This avoids permanent changes in host organisms (Pickar-Oliver and Gersbach, 2019). Although protein targeting strategies, such as monoclonal antibodies, are a popular therapeutic approach offering similar advantages, protein therapeutics are limited by 'druggable' targets: only 1.5% of the human genome encodes for protein while 70% encodes for non-coding RNAs (ncRNAs). Targeting RNA thus significantly broadens therapeutic targets (Warner et al., 2018). In addition, targets may be specified simply by knowledge of the target RNA sequence. Recognising these benefits, RNA engineering was recently reported as a promising candidate to become one of the most impactful advances for science in the 21st century (Thavarajah et al., 2021).

ASO and RNA interference (RNAi) are the two strategies that have been clinically employed for RNA-targeted therapeutics. Using these strategies, 12 drugs have been developed and approved to date for various genetic diseases (**Table 1**) (Winkle et al., 2021). While offering promise, manufacturing drugs based on these strategies continue to be complex and a cautious approach is still required with ASO and RNAi strategies due to off-target effects that may affect other essential pathways. Delivery is also challenging due to poor cellular transduction and cytotoxicity, requiring carrier proteins or chemical modifications for therapeutic development (Roberts et al., 2020).

In 2012, the description of a programmable gene editing platform transformed biotechnology (Jinek et al., 2012). Known as clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR associated (Cas), the technology allowed designing RNA strands that could target specific DNA sequences for cleavage. Subsequent research has led to the discovery of exclusively RNA-targeting CRISPR-Cas systems, such as CRISPR-Cas13. While ASOs have not been experimentally compared with CRISPR-Cas13 systems yet (Palaz et al., 2021), these systems have exhibited enhanced efficiency and specificity over RNAi (Abudayyeh et al., 2019; Cox et al., 2017; Zhang et al., 2021b). CRISPR-Cas13 systems have so far been demonstrated for knockdown, multiplexed targeting, base editing, and demethylation applications across genetic and infectious diseases (Chuang et al., 2021b; Cox et al., 2017; Xie et al., 2021). Importantly, CRISPR-Cas13 systems now allow for all these applications to be achieved through delivery from a single-AAV vector.

In this review, we describe emergence of the existing RNA-targeting strategies, their applications in ocular disease and the current challenges. Then, we discuss the emergence of CRISPR-Cas and the recent RNA-targeting CRISPR-Cas systems, their potential in addressing ocular disease and the solutions they offer. Lastly, we look at the considerations for developing and commercialising novel therapeutics to inform future ocular gene therapy efforts.

This review is focused on RNA editing for ocular disease. For a general overview of small molecule, gene or cell therapies for ocular disease, we refer the reader to recent excellent reviews on therapeutics against acquired ocular diseases (Gagliardi et al., 2019; Lin et al., 2020; Tan et al., 2021) and inherited ocular diseases (Britten-Jones et al., 2022; Fenner et al., 2022; Schneider et al., 2021).

2 A brief history of RNA and its many functions

RNA was discovered in the 1890s, differentiated from DNA through localising in the cytoplasm and containing ribose sugars (Allen, 1941). It was generally thought to only function in ribosomes for the translation of proteins as ribosomal RNAs (rRNA). This understanding transformed after the

description of an unstable RNA intermediate that facilitates protein synthesis, now commonly known as messenger RNA (mRNA) (Brenner et al., 1961; Gros et al., 1961). During this time, the discovery of transfer RNA (tRNA) also showed that RNA was involved in binding amino acids, exposing the diverse and crucial functions played by RNA in protein synthesis (Hoagland et al., 1958). Later, RNA polymerase was described in 1962 to be responsible for RNA biosynthesis (Furth et al., 1962). Isolated from *Escherichia coli*, RNA polymerase was dependent on the DNA template to synthesize RNA. Put together, the 'Central Dogma of Biology' was established as DNA → RNA → Protein (Crick, 1970).

As the dogma suggests, RNA was merely thought of as an intermediate in protein synthesis. ncRNAs were therefore a surprising discovery. In 1971, 'trimming' and 'tailoring' was suggested in the process of transcription to explain the presence of rRNAs and tRNAs (Burdon, 1971). The author described these events as maturation and reported how regions of tRNA and rRNA were discarded to form functional RNA. The structure of mRNA provided the first clues to explain the phenomenon, with polyadenylated tails and 5' caps (Darnell, 1976). The breakthrough came when, in 1977, scientists showed how regions from the adenoviral genome were put together to make mRNA (Berget et al., 1977; Chow et al., 1977), elucidating splicing of mRNA. In addition, the RNA structure of introns were observed to be essential in achieving splicing (Kruger et al., 1982). Alongside, RNase P was characterised to require a protein subunit and an RNA molecule to produce mature tRNAs (Guerrier-Takada et al., 1983). This led to the understanding that RNA could perform enzymatic functions as well, which are now commonly known as ribozymes. It is now known that RNA plays a myriad of roles other than coding for protein, for instance, long non-coding RNAs (lncRNAs) that may control transcription, function as molecular decoys or aid protein folding. Various forms of RNA have been characterized with diverse functions and are excellently reviewed elsewhere (Cech and Steitz, 2014).

3 Antisense oligonucleotides (ASO)

ASOs are short (12-24 nt) single-stranded nucleic acids (DNA or RNA) programmed to bind to specific complementary mRNA targets through Watson-Crick base-pairing for the modulation of gene expression. ASOs function through inhibiting natural gene expression processes, that have been repurposed for therapeutic applications In 1977, translational activity was shown to be inhibited through hybridisation with complementary DNA in a cell-free system (Paterson et al., 1977). Subsequently, virus replication was inhibited using a DNA sequence antisense to respiratory syncytial virus (RSV) RNA (Zamecnik and Stephenson, 1978). In 1983, *trans* inhibition of translation was reported through "direct pairing between transposase messenger RNA and a small complementary,

regulatory RNA" (Simons and Kleckner, 1983). Antisense regulation of genes is now well understood in both prokaryotic and eukaryotic systems and are covered elsewhere (Crooke et al., 2021).

For therapeutic uses, ASO therapy can be divided into either RNAse-H dependent ASOs that recruit catalytic enzymes for RNA cleavage, or steric blockade ASOs that block binding of *trans*-acting splicing or translation factors (Uhlmann and Peyman, 1990). The first FDA approved ASO treatment was for ocular disease in 1998. Fomivirsen (or Vitravene®) was used to treat CMV retinitis (Geary et al., 2002), targeting the mRNA of CMV genes that are essential for viral replication, thereby reducing immediate-early protein synthesis (Azad et al., 1993). Unfortunately, Fomivirsen was eventually discontinued by the manufacturer due to lack of demand.

For effective therapeutic use, ASOs should be able to enter and be retained in cells to interact with their target in a sequence-specific manner. Poor stability, adequate intracellular penetration and adequate bioavailability are notable considerations that have challenged the development of ASOs as therapeutics. Various modifications to ASOs have been made for different applications to address these challenges. ASOs may come in many forms such as ssDNA, phosphorothioate DNA, RNA analogues, conformationally restricted nucleosides or morpholino phosphorodiamidate oligonucleotides. As ASO technology, its mechanism of action and clinical applications have been thoroughly reviewed recently, we refer the reader to these articles for a comprehensive understanding of ASOs (Crooke et al., 2021; Crooke et al., 2020a; Crooke et al., 2020b). Here, we only briefly describe ASO mechanism of action (**Figure 2**), before discussing their applications in ocular disease.

3.1 ASO mechanism of action

ASOs function through complementary pairing with a designated target RNA (Baker et al., 1997; Wu et al., 2004). ASOs may function through RNase H-dependent or RNase-independent mechanisms (Dias and Stein, 2002).

It was established in 1979 that RNase H can mediate degradation of target RNA after ASO hybridisation (Donis-Keller, 1979). Specifically, RNase H functions to hydrolyse RNA in RNA-DNA hybrids (Stein and Hausen, 1969), a process that requires divalent cations, like Mg^{2+} or Mn^{2+}, to produce end products with a 5' phosphate group and 3' hydroxyl group (Wu et al., 2004). ASO-RNA duplexes act as substrates for RNase H to bind and perform RNA hydrolysis. Of two RNase H enzymes in humans, RNase H1 and RNase H2, the former is reported to be critical (Wu et al., 2004), and found in the nucleus and cytoplasm, while RNase H2 is only found in the nucleus (Liang et al., 2017). RNAse H dependent cleavage is often preferred to the independent alternative, and thus modifications are

tailored to enable the recruitment of RNase H. Gapmers are one such example. Generally speaking, gapmers are 16-20nt in size, consisting of a central 8-10nt DNA-based region to promote DNA-RNA hybrid degradation, flanked on both sides by 4-5nt RNA-based, chemically modified region to promote target binding (Scharner and Aznarez, 2020). Initially, gapmers without modifications were readily degraded by nucleases and thus unstable. A significant advance was made with phosphorothioate modifications that prevented nuclease degradation and allowed enhanced binding to various proteins for facilitating delivery, ASO release and subcellular distribution (Clercq et al., 1969; Crooke et al., 2020b). Subsequent sugar modifications have also been identified to improve gapmer safety and activity (Shen et al., 2019; Vasquez et al., 2021).

Alternatively, ASOs can also achieve RNA modulation through sterically blocking translational machinery or splicing factors. Typically composed only of modified bases, these ASOs lack DNA bases, and therefore are not recognised or degraded by RNAse H mechanisms. Steric blockade of transcription factor binding can be used achieve exon exclusion or can be used to halt protein synthesis through preventing ribosome binding to mRNA and arresting polypeptide chain elongation (Dias et al., 1999; Dias and Stein, 2002). Modulation of splicing can also lead to intron inclusion, resulting in production of immature proteins. Modified oligonucleotides such as 2'-O-alkyl, peptide nucleic acid (PNA) and morpholino ASOs allow these varied forms of inhibition.

3.2 ASO therapies against ocular disease

ASOs were investigated for development of ocular therapeutics starting from the early 1990s. The first use of ASO therapy for an ocular condition was described in 1994, targeted at insulin growth factor I (*IGF-I*) to inhibit conditioned eye blink response in rats (Castro-Alamancos and Torres-Aleman, 1994). Soon, in 1996, delivery of ASO to the retina was being discussed (Rakoczy et al., 1996). From the early 2000s, ASO therapies have been developed for neovascular, inherited and orphan ocular diseases (**Table S1**).

In targeting neovascular disease, vascular endothelial growth factor (*VEGF*) has been a common target in preclinical studies for ASO therapeutic development, targeting choroidal, corneal and iris neovascularisation, however none have yet to progress to clinical trials. Choroidal neovascularization was also one of the first ocular diseases for which ASO therapies were investigated. Efficient inhibition of *VEGF* and neovascularization was demonstrated *in vitro* and *in vivo* in rat models with this pioneering study (Garrett et al., 2001). ASO therapy to target inflammatory factors and address

keratitis, chorioretinitis and inflammation following glaucoma surgery were evaluated throughout the 2000s (Cordeiro et al., 2003; Mei et al., 2009; Wasmuth et al., 2003).

More recently, ASO therapy for targeting inherited retinal degenerations (IRDs) has been investigated. ASO therapy for LCA was studied in 2012 in two studies, both targeting the c.2991+1655A→G mutation in *CEP290* (Collin et al., 2012; Gerard et al., 2012). The c.2991+1655A→G mutation leads to insertion of a cryptic splice site that causes premature termination of *CEP290*, and reduced protein levels. The authors showed that ASO therapy could direct normal splicing of *CEP290*, increase levels of wildtype *CEP290*, reduce mutant protein and recover the ciliation process. Since then, ASO therapy has been tested for other inherited conditions like retinitis pigmentosa, Stargardt disease and Usher syndrome (Sangermano et al., 2019; Slijkerman et al., 2016). Most recently, the *USH2A* gene was targeted for treatment of retinitis pigmentosa, demonstrating exon skipping in patient-derived iPSC cells and mouse models (Dulla et al., 2021).

Several ASO therapies have progressed to clinical trials (**Table 2**). Notable among them is QR-110 (or sepofarsen) for treatment of LCA, for which phase I studies (NCT03140969), showed no severe side effects, with improved vision at 3 months (Cideciyan et al., 2019) and sustained visual gain at 15 months in one patient (Cideciyan et al., 2021). QR-110 has now progressed to Phase II/III trials (NCT04855045 and NCT03913143), however recent results made public by the company indicate this trial unfortunately did not meet its primary end points of improvement in BCVA or other secondary endpoints, with formal publication of the trial results still to come (https://www.proqr.com/community-stories-and-news/phase-23-illuminate-trial-results-of-sepofarsen-in-cep290-mediated-lca10). GS-101 (or Aganirsen) was also slated for Phase III trials for treatment of ocular neovascularisation, however no updates on the study are available (NCT02947867).

3.3 ASO delivery strategies

While ASO modifications improve their deliverability and stability, delivery across the lipid bilayer remains a challenge. Furthermore, due to eventual ASO degradation, repeated administrations are required for sustained effect. Viral or non-viral vectors are thus appealing for ASO delivery to overcome these challenges (Juliano, 2016; Xue and Maclaren, 2020).

Delivery through vectors helps intracellular and intranuclear uptake of ASOs and improve access to mRNA targets. For example, ASOs can be delivered within a cationic nano-emulsion for treatment of corneal neovascularisation in rat and mice models (Hagigit et al., 2012). The cationic nanoemulsion delivery, being made of positively charged nanodroplets, adheres well to the negatively charged cornea

surface, allowing enhanced uptake of drug. Delivery within the cationic nanoemulsion achieved an 80% inhibition of *VEGF* expression, twice the rate achieved when delivered in control vehicle. Other genes that have been targeted using ASOs delivered through cationic lipids and shown efficient knockdown are the antiapoptotic proteins Bcl-2, Bcl-xL, and intercellular adhesion molecule I (*ICAM-1*) (Lebedeva et al., 2000). Toxicity, however, has been reported with the use of cationic lipids, through interaction with cell membrane and disruption of membrane function (Teixeira et al., 2017). Proteins with the capacity to penetrate cell membranes can also be conjugated with ASOs for improving delivery. The most common protein conjugate is that of poly-L-lysine (PLL) along with a carrier for binding cell surface receptors. Examples of carriers include insulin, lectins and glycoproteins. Association with block polymers to form polyionic micelles has also been shown to be efficient in carrying ASOs to their target (Lebedeva et al., 2000).

Viral vectors are generally not preferred for ASO delivery due to the numerous options available with nanocarriers or highly modified naked ASOs. One study used adeno-associated virus AAV2/9 to deliver ASO *in vivo* targeting the *CEP290* splicing mutation, c.2991 +1655A>G, for treatment of LCA. Compared to delivery of naked ASOs, AAV delivery resulted in poorer rescue of correct splicing. However, AAV delivery of ASO still resulted in statistically significant recovery of the correctly spliced gene (Garanto et al., 2016).

4 RNA interference (RNAi)

RNAi pathways regulate gene expression by the modulation of the stability and translation of mRNA in cells by sequence-specific double stranded RNA. The mechanisms of post-translational gene silencing were described in the nematode worm (*Caenorhabditis elegans)* in 1998 when the introduction of dsRNA resulted in the silencing of an endogenous gene (Fire et al., 1998), and termed RNAi. Soon, RNAi was developed into one of the most diversely applicable tools (Elbashir et al., 2001), providing researchers with an additional tool to develop therapeutics (Saw and Song, 2020). A method to stably express siRNAs in mammalian cells was described in 2002 (Brummelkamp et al., 2002), setting the path for RNAi-mediated therapeutics.

4.1 RNAi mechanism of action

RNAi functions through double-stranded small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs), that is complementary to the target RNA. Unlike single-stranded ASOs, which can bind

directly to a target RNA, the double-stranded siRNAs must be processed prior to RNA binding. These siRNAs are initially cleaved from longer double stranded pre-siRNAs by the Dicer protein, to produce short fragments of around 20 nucleotides in length. siRNAs at this stage are double stranded, and associate with the RNA-induced silencing complex (RISC), a multiprotein complex, which contains argonaute 2 (Ago2) protein. The Ago2 protein is the active molecule of RISC. One strand of siRNA, called the passenger strand, is degraded by PIWI domain of the Ago protein. The remaining strand then guides the RISC complex to target mRNA, by complementary base pairing, for cleavage by Ago protein (**Figure 3**). RNAi may also work with miRNAs and piwi-interacting RNAs (piRNAs), however therapeutics are generally based on siRNA and less commonly with shRNAs if genome integration is preferred (Wang et al., 2019).

4.2 RNAi in ocular disease

The emergence of RNAi created a competitor to ASOs (Jones and Schreiber, 2005) (**Table S2**). As with ASOs, VEGF was the first target for neovascular diseases in the early 2000s. In one notable study, multiple siRNAs targeting VEGF, vascular endothelial growth factor receptor-1 (*VEGFR1*) and *VEGFR2* resulted in higher inhibition of neovascularization than any one siRNA alone, both *in vitro* and in mice (Kim et al., 2004). Importantly, the study showed multiplexing siRNAs as a therapeutic against ocular angiogenesis for the first time. The rhodopsin (*RHO*) gene was subsequently targeted as a treatment for one form of retinitis pigmentosa (RP). For example, a study found siRNA targeting *Rho* in mice to be efficient at suppressing mRNA levels, but remain clinically inefficient (Tessitore et al., 2006). Mutational heterogeneity presents a significant challenge when targeting IRD genes, such as *RHO*, where over 100 mutations may be involved in disease condition. To overcome this barrier, researchers have employed the suppression and replacement strategy. Here, both wildtype and mutant alleles are suppressed by RNAi in a mutation-independent manner, and a replacement gene, resistant to suppression, is delivered in tandem (O'Reilly et al., 2007). Several preclinical studies have demonstrated a therapeutic benefit of this strategy as a treatment for autosomal dominant retinitis pigmentosa (adRP) (Cideciyan et al., 2018; Mao et al., 2012; Millington-Ward et al., 2011). The balance between suppression and replacement components must, however, be carefully determined to reduce toxicity and the risk of unintended off-target effects on transcripts and bystander cell populations. These considerations have slowed the translation of pre-clinical studies towards the clinic. Recently, RNAi with artificial mirtrons, which are miRNAs within introns of mRNA-encoding genes, have been demonstrated to overcome these concerns in targeting adRP (Orlans et al., 2021). Mirtrons may be expressed using cell-specific promoters, thereby limiting toxicity and off-target effects. In mice

models with a heterozygous proline to histidine knock-in mutation at position 23 (P23H) that present rapid with retinal degeneration, suppression and replacement therapy with artificial mirtrons through subretinal injection resulted in slowing of retinal degeneration (Orlans et al., 2021).

For glaucoma, an siRNA therapeutic targeting the *CASP2* gene presented promising results in terms of safety, retinal ganglion cell (RGC) survival and prolonged neuroprotection of up to 30 days in rats (Ahmed et al., 2011; Solano et al., 2014). The most recent study has targeted placental growth factor (*PlGF)* gene for treatment against neovascular disease (Araújo et al., 2020).

With regards to RNAi therapies in clinical trials (**Table 3**), Tivanisiran eye drops has completed phase III trials in 2020 for treatment of dry eye disease, showing improvement in all main symptoms of the disease (Gonzalez et al., 2020). Bevasiranib for treatment of neovascular age-related macular degeneration (nAMD) has also progressed to phase III trials, however, severe adverse effects, such as decreased visual acuity and endophthalmitis, have resulted in the termination of one clinical trial (NCT00499590), and another was never initiated (NCT00557791). Another Phase III trial targeting *CASP2* for glaucoma was also terminated for unknown reasons (NCT02341560).

4.3 siRNA delivery strategies

Delivery of siRNA can be done locally or systemically, depending on the target cell and tissue. Delivery to the eye, skin or muscle is easy to access through local delivery.

Modifications to siRNA can help evasion of immune reactions and provide resistance to endonucleases like that of ASO modifications. 2'-O-methyl modifications, introducing phosphorothioate backbone linkages, conjugation to peptides, lipids or polyethylene glycol (PEG) are applicable to siRNAs as well (Whitehead et al., 2009). Where naked or modified siRNAs are inefficient, nanoparticles can be used to improve biodistribution. The various methods available for non-viral siRNA delivery have been reviewed in detail elsewhere (Liu et al., 2021). Interestingly, siRNA has been delivered through a nanoball targeting *VEGF* mRNA. Composed of siRNA hydrogel, branched polyethylenimine and hyaluronic acid, the nanoball was administered through intravitreal administration and achieved up to 61% inhibition of *VEGF* mRNA, showing potential as therapeutic for choroidal neovascularization (Ryoo et al., 2017).

Adenoviruses, lentiviruses and retroviruses have all been demonstrated for viral delivery of siRNAs. Viral vectors have excellent efficiency with tissue-specific tropism, but safety concerns persist with genomic integration and immunogenicity (Shim and Kwon, 2010). AAV vectors have not been

extensively studied with developing RNAi therapeutics for ocular diseases, but one study has shown ~80% silencing efficiency of *GCAP1* gene in mice with self-complementary AAV (scAAV)2/8 delivery of siRNA for treatment of retinitis pigmentosa (Jiang et al., 2011).

5 CRISPR-Cas gene editing

While programmable CRISPR-Cas gene editing was described less than a decade ago (Jinek et al., 2012), CRISPR is an ancient adaptive immune mechanism evolved in bacteria and archaea.

In 1987, unique 'spacer' sequences flanked by repeat sequences were reported (Ishino et al., 1987). Recognizing these spacers as identical to viral sequences revealed that bacteria derive these spacers directly from the viruses that infect them to develop 'vaccination cards' against subsequent infection (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005). Essentially, these spacers encode for CRISPR RNA (crRNA) that targets the complementary viral sequences. Upon association with the target sequence, a Cas enzyme is recruited for cleavage, thereby protecting the bacterial host from infection. Two classes of CRISPR-Cas systems exist, namely class 1 and class 2. Under class 1 are the CRISPR-Cas systems with multidomain effectors (Types I, III and IV). Types II, V and VI come under class 2 CRISPR-Cas systems with single domain effectors, and consist of the Cas9, Cas12 and Cas13 enzymes, respectively. The different types of CRISPR-Cas systems can be further classified into subtypes (e.g. II-A, VI-B) based on their host organism (Makarova et al., 2020). Generally, Cas enzymes are referred to by their type and subtype. For example, Cas9 from *Streptococcus pyogenes* is known as SpCas9, whereas that from *Staphylococcus aureus* is known as SaCas9. Studies of CRISPR loci have revealed adjacent AT-rich leader sequences serving as promoter elements. Put together, a CRISPR loci generally consists of Cas genes, a leader sequence, and alternating spacer and repeat sequences. The development of CRISPR-Cas9 as a programmable gene editing platform (Doudna and Charpentier, 2014), and experimental design and delivery methods have been well described (Burnight et al., 2018).

5.1 RNA-targeting CRISPR-Cas systems

Despite the versatility of CRISPR-Cas9 systems, their specificity for DNA sequences was a glaring limitation. The possibility of RNA-targeting CRISPR systems, however, was alluded to in 2009 through an RNA silencing system present in prokaryotes that worked against invader viruses using invader-derived sequences for homology-dependent cleavage (Hale et al., 2009). Later, as binding to

target sequence was based on protospacer adjacent motif (PAM) sequence for Cas9-mediated cleavage, scientists stimulated site-specific Cas9 cleavage of ssRNA by presenting the PAM sequence separately as PAM presenting oligonucleotides (PAMmers) (O'Connell et al., 2014), developing an RNA-targeting Cas9 (RCas9) system. A truncated RCas9 compatible with AAV vectors was subsequently developed (Batra et al., 2017).

Cas9 orthologues, such as SaCas9 and CjCas9, can target RNA *in vitro* without requiring a PAM sequence. Further study with SaCas9 showed that it could provide a protective effect against RNA phage infection in bacteria. SaCas9 RNA-targeting activity is, however limited to ssRNA of low structural complexity and structured RNAs are not cleaved by SaCas9, limiting widespread applicability (Strutt et al., 2018). *Franscisella novicida* Cas9 is also capable of targeting bacterial mRNA (Sampson et al., 2013), and has been repurposed to target the human hepatitis C virus, an ssRNA virus, in eukaryotic cells (Price et al., 2015). FnCas9 is also PAM-independent, not requiring PAMmers.

Further study of Type V effectors revealed the RuvC domain of Cas12g to be distinct with the ability to cleave ssRNA without requiring a PAM sequence. In the presence of RNA, Cas12g could also cleave ssDNA in an unspecific manner (Tong et al., 2021a; Yan et al., 2019). The type III CRISPR effectors, Csm and Cmr complexes, target both DNA and RNA, however, the multiple subunit composition can make these systems prohibitively complex for RNA targeting therapeutics. (Wang et al., 2019). Comparatively, the single domain Type VI CRISPR effectors such as Cas13 have shown promise for therapeutic RNA targeting applications.

5.2 Discovery of CRISPR-Cas13

In the search of novel CRISPR systems, Shmakov *et al.* searched for the presence of Cas1 was searched across the NCBI whole genome shotgun sequence database using a computational pipeline (Shmakov et al., 2015) Large uncategorized proteins were then filtered to identify novel CRISPR loci (O'Connell, 2019). This led to the discovery of type VI CRISPR-Cas13 enzymes, that marked a leap in RNA-targeting systems due to their simplicity and exclusive RNA-targeting properties. Specifically, Cas13 endonucleases cleaved only ssRNA, and not ssDNA, dsDNA, or dsRNA (Abudayyeh et al., 2016; East-Seletsky et al., 2016; Smargon et al., 2017). Most importantly, they possessed much lower rates of off target editing in eukaryotes (Ali et al., 2018). Recent reviews comprehensively outline the structure, functions and applications of CRISPR-Cas13 systems (Kordyś et al., 2021; Perčulija et al., 2021), and another specifically details its versatile applications for epitranscriptional editing, where

chemical modifications are made in RNA to modulate translation, splicing or translocation (Tang et al., 2021b). To date, six Cas13 enzymes have been described (Cas13a, Cas13b, Cas13c, Cas13d, Cas13X and Cas13Y) (**Table S3**).

The latest Cas13X and Cas13Y are of particular interest due to their compact size (Xu et al., 2021). They range 775aa to 805aa in size, the smallest Cas13 enzymes to date. Cas13X consists of two variants, Cas13X.1 and Cas13X.2, sized 775aa and 805aa respectively. Five variants come under Cas13Y, Cas13Y.1 to Cas13Y.5, ranging 790 to 803aa. Cas13X.1 showed the highest knockdown efficiency among the seven new identified effectors, when the mCherry reporter was targeted. RNA knockdown was crRNA and HEPN-dependent, confirmed through using nontargeting crRNA and inactive Cas13 respectively.

5.3 CRISPR-Cas13 mechanism of action

In Cas13 systems, the HEPN domains have dual functions: crRNA maturation and target RNA cleavage (**Figure 4**). For example, in LshCas13a, Helical-1 domain in the recognition (REC) lobe and in LbuCas13a, the HEPN2 domain in the nuclease (NUC) lobe is involved in pre-crRNA maturation to crRNA. Depending on Cas13 system, pre-cRNA is cleaved with DR in 5' or 3' end. Types VI-A, VI-C and VI-D have their repeats at the 5' end, while VI-B, VI-X and VI-Y have direct repeats (DR) in the 3' end (Smargon et al., 2017; Xu et al., 2021). It has however been shown that crRNA maturation is not strictly required for catalytic activity (East-Seletsky et al., 2017). Upon binding with target ssRNA, the Cas13 complex undergoes a confirmational change, with the HEPN domain moving closer to each other to form a single catalytic site. The catalytic site then cleaves the target and other ssRNA in proximity (Liu et al., 2017). PFS requirements are also dependent on the type of Cas13 system. VI-D and VI-X systems have no bias for PFS sequences, while LshCas13a prefers a non-G base at 3' end and BzCas13b requires a non-C base immediately upstream of target site and a NAN or NNA sequence 3' of target site (Abudayyeh et al., 2016; Smargon et al., 2017).

5.4 Delivery strategies of CRISPR-Cas system

Efficient delivery of the CRISPR-Cas system to target organs is essential for therapeutic purposes. We recently reviewed the *in vivo* delivery strategies and challenges for the CRISPR-Cas system (Chuang et al., 2021a). The CRISPR-Cas complex (Cas enzyme and gRNA) can be delivered as DNA, RNA,

or protein molecules. Most commonly, the CRISPR-Cas complex is introduced as DNA encased in viral vectors.

Indeed, we were the first group to demonstrate viral delivery of CRISPR-Cas in the retina, and showed safe and efficient transduction by AAVs (Hung et al., 2016). The strategy incorporated SpCas9 with AAV2 vectors, which was co-delivered intravitreally with guide RNA targeting YFP into transgenic Thy1-YFP mice. This dual-AAV CRISPR-Cas system achieved 84% knockdown of YFP-expressing cells in the retina. While AAV2 expression predominated in the ganglion cell layer, a 50% YFP knockdown was observed in RGCs. We further studied the effect of expressing the CRISPR constructs through AAVs in the retina. No adverse effects on retinal function were observed in photoreceptors or bipolar cells through ERG. Inner retinal function and ganglion cell activity was also unperturbed. OCT revealed no significant difference in retinal nerve fiber layer (RNFL) and ganglion cell complex (GCC) thickness compared to control (**Figure 5**). Further data evaluating the long term expression of AAV-CjCas9 construct with a gRNA targeting *Hif1a* found no adverse electroretinographic or histological effects in mice at 14 months post-intravitreal injection (Jo et al., 2019). Among viral vector delivery methods, AAVs remain a leading candidate for *in vivo* CRISPR-Cas delivery, possessing a strong track record of safety in clinical trials and the capability to efficiently transduce multiple organs and cells (Wang et al., 2020a).

While AAV-mediated delivery is commonly preferred for gene therapy, large constructs such as CRISPR-Cas are challenging to deliver efficiently using AAVs. Large genes or editing constructs can be delivered using two or more AAVs with the gene cargo split across the viruses. Dual-vector strategies include delivery the guide RNA on a separate vector to the Cas (Hung et al 2016) or splitting of the Cas transgene across two AAVs so that the transgene or the protein products are recombined intracellularly to produce a full-length protein. For example, a base editor divided across two AAVs and recombined using a split-intein approach demonstrated efficient base editing in both neurons and the retina (Levy et al., 2020). Dual AAV strategies often are associated with reduced full-length transgene expression or with reduced editing efficiency (Carvalho et al., 2017), and can require significant engineering and optimisation to overcome these obstacles. Different AAV serotypes also vary in their tropism and must be considered when targeting different organs and cell types (Korneyenkov and Zamyatnin, 2021). In a currently active clinical trial (NCT03066258), AAV8 is used to deliver an anti-VEGF agent to the retina. Preclinical studies to determine transduction capabilities of AAV serotypes is tantamount.

Other viral delivery vectors such as lentiviruses and adenoviruses may also be used. These vectors allow for a larger cargo capacity (8-10kb) unlike AAVs. However, lentiviruses integrate into the host

genome, while adenoviruses may be pathogenic and induce a strong immune response. Non-viral delivery methods like electroporation, liposomes, and nanoparticles have also been extensively studied. Non-viral vectors generally have a poorer transduction profile compared to the viral alternatives; however, they do allow delivery of ribonucleoproteins (RNPs). Delivering CRISPR-Cas RNPs allow for fast turnover of the Cas enzyme, reducing risk from off-targets, while maintaining comparable editing efficiency. It also allows more control over Cas expression as host cell protein expression is not involved (Yu and Wu, 2020).

Alternatively, targeting RNA rather than DNA may also offer safer gene editing tools. As RNA edits are impermanent, prolonged expression of CRISPR-Cas constructs pose a significantly lesser risk. The need for self-destructing constructs also becomes obsolete.

6 CRISPR-Cas13 for ocular disease management

6.1 RNA knockdown with CRISPR-Cas13

While limited, studies with CRISPR-Cas13 against ocular disease have shown promise for the development of therapeutics for glaucoma and neovascular disease.

We recently described methods for designing CRISPR-CasRx-based (RfxCas13d) knockdown experiments, and demonstrated knockdown of *VEGFA* mRNA *in vitro* using single vector system (Chuang et al., 2021b). In addition, we also showed efficient knockdown *in vitro* through delivery of pre-sgRNAs as gBlocks™, eliminating the need for cloning. Furthermore, multiplexed knockdown was demonstrated with three pre-sgRNAs incorporated with CRISPR-CasRx in a single AAV vector in HEK293FT and Müller cells. This also demonstrates the feasibility of targeting multiple genetic factors that contribute to angiogenesis for enhanced therapeutic effect, such as coupling VEGF and PDGF knockdown (**Figure 6A**). In addition to showcasing the RNA knockdown efficiency of Cas13 enzymes, our study also demonstrated the feasibility to incorporate CRISPR-Cas13 system in a single AAV vector for therapeutic delivery. This ensures safe and long-term RNA editing, due to non-integrating and persistent nature of AAVs (**Figure 6B**).

Subsequently, following studies that showed downregulation of polypyrimidine tract-binding protein 1 (*Ptbp1*) to convert mice fibroblasts into functional neurons (Xue et al., 2013), the CRISPR-CasRx system was employed to target *Ptbp1* (Zhou et al., 2020b).. This effectively downregulated *Ptbp1* to stimulate the conversion of Müller glia to RGCs and replenish RGCs in an N-methyl-D-aspartate (NMDA)-induced RGC injury model with partial restoration of visual responses and vision-dependent

behaviour. This approach may have therapeutic applications in conditions like glaucoma where RGC loss leads to degeneration of optic nerve.

Using AAVs to achieve persistent and safe RNA editing has been further demonstrated in another notable study (Zhou et al., 2020a). Here, CRISPR-CasRx also used to study the effect of VEGFA knockdown in mice with choroidal neovascularisation (CNV). CNV is a hallmark of wet AMD, and the leading cause of blindness in adults over the age of 50. Delivery of CasRx through AAVs to target *VEGFA* mRNA significantly reduced *VEGFA* mRNA and VEGFA protein levels, 22.7% and 68.2% respectively, as compared to control eyes. CNV area was also reduced in the treated mice models (Zhou et al., 2020a). With this study, the authors have demonstrated the feasibility for developing a compact, potent, and simple gene editing strategy for ocular neovascularisation. The small size of CasRx was exploited to develop a single vector CRISPR-Cas construct. Significant levels of mRNA knockdown were achieved *in vitro*, and *in vivo*, and clinically significant effects were observed. While early studies reported Cas13 systems to not exhibit collateral cleavage in eukaryotic cells (O'Connell, 2019), recent studies have shown otherwise where Cas13a, Cas13c and Cas13d have all exhibited collateral activity in HEK293FT and U87 cells (Özcan et al. 2021). Nevertheless, the small size of newer Cas13 enzymes and their enhanced specificity offer attractive opportunities for therapeutic development. In addition, multiplexed gene knockdown can also be performed through a single AAV vector, currently the most efficient *in vivo* delivery method for gene therapy.

As several clinical and preclinical studies have targeted causative genes of common ocular diseases for gene silencing, RNA silencing with CRISPR-Cas13 can be conceived for the same to develop safer gene therapies. We have therefore compiled these ocular conditions and their causative genes to inform future preclinical studies using CRISPR-Cas13 knockdown (**Table 4**). However, it is important to note that AAV-mediated Cas13 mRNA knockdown will produce a persistent gene knockdown and would be detrimental for therapeutic purposes. For example, complete knockout of *VEGFA* in retinal pigment epithelium (RPE) cells would lead to degeneration of choriocapillaris (Marneros et al., 2005). In this case, an inducible RNA knockdown system (e.g., inclusion of destabilising domains) would be appropriate (Chen et al., 2021).

6.2 Deactivated Cas13 and Cas13-effector fusions enable versatile strategies for RNA therapeutics

In addition to cleavage and knockdown of RNA, CRISPR-Cas13 enzymes can be delivered with a multitude of effectors for other therapeutic purposes. Through mutations in the HEPN domains of Cas13 enzymes, catalytic activity can be inactivated while retaining crRNA processing and RNA

targeting (Cox et al., 2017; Konermann et al., 2018). These deactivated Cas13 molecules therefore can be directed to target bind to specific RNA transcripts without cleavage. These have been used for modulation of splicing and RNA methylation, as well as RNA base editing (described in further detail in section 6.3)

6.2.1 Splicing modulation

Deactivated Cas13 has been used to manipulate splicing of RNA in an approach similar to ASO modulation. Using multiplexed guides targeting regulatory sequences important for splice site recognition, exon skipping can be induced in human transcripts (Konermann et al., 2018). Fusion of the negative splice factor hnRNPa1 to dCasRx was also shown to improve rates of exon skipping *in vitro* (Konermann et al., 2018). In iPSC-derived cortical neurons from frontotemporal dementia patients, an AAV-delivered dCasRx exon skipping approach has been used to induce therapeutic levels of exon 10 exclusion in the gene *MAPT* to alleviate dysregulated 4R/3R tau ratios (Konermann et al., 2018).

6.2.2 Epigenomic/epi-transcriptomic regulation

Epigenome editing is being explored with enthusiasm currently due to significant linkages between epigenetic state and phenotype. Specific alteration of cellular epigenome can reveal the contribution of epigenetic states in disease progression and allow precise manipulation of biological function (Nakamura et al., 2021). RNA methylation of adenosine to form N^6-Methyladenosine (m^6A) is the most common endogenous RNA modification and is known to affect splicing, nuclear export, stability of RNA as well as translation efficiency. In humans, this process is mediated by methyltransferase like 3 (METTL3) and methyltransferase like 14 (METTL14). Fusion of METTL3 and METTL14 with catalytically inactive PbuCas13b allows targeted RNA methylation in both the nucleus and cytoplasm. Low off-target activity was also observed with dCas13b-METTL3 localised to the nucleus (Wilson et al., 2020). Furthermore, dCas13d was recently used for m^1A demethylation, by coupling Cas13 with RNA demethylase, developing a strategy known as REMOVER (for *reengineered m1A modification valid eraser*) (Xie et al., 2021). Epitranscriptome screening can also reveal causal factors in disease state, and development of Cas13 systems for this purpose allows us to study effects of endogenous RNA modification.

6.3 RNA base editing

Base editing is an emerging frontier of CRISPR gene editing that is garnering much current interest (Abudayyeh et al., 2019; Cox et al., 2017; Gaudelli et al., 2017; Komor et al., 2016). Base editing refers to targeting single base mutations that have been identified to be causative for disease state. For CRISPR, this can be achieved through exploitation of Cas enzymes' DNA/RNA targeting ability. Adenosine or cytosine deaminases have been fused with inactive Cas enzymes to perform A→G or C→T base editing. Deaminase domains differ based on application, for example APOBEC or TadA deaminases are used for base editing in DNA (Chu et al., 2021; Xie et al., 2020), while ADAR enzymes are used for RNA base editing (Abudayyeh et al., 2019; Cox et al., 2017).

Remarkable work has been done with DNA base editors, predominantly from the David Liu group, to achieve all base exchanges (C→T, T→C, A→G, and G→A) without causing double strand breaks (Gaudelli et al., 2017; Komor et al., 2016). Concerns over the permanent nature of potential off-target mutations due to DNA editing have led to an increased interest in RNA editing as a therapeutic approach (Zhang et al., 2021a). Off-target effects throughout the transcriptome have also been observed with DNA base editors (Grünewald et al., 2019). Nevertheless, studies have reported efficient DNA base editing in retinal genes along with recovery of phenotype, showing their potential in treating ocular disease (Choi et al., 2022; Suh et al., 2021).

RNA editing occurs naturally as a post-transcriptional process through changing nucleotide composition of RNA to alter function of proteins or regulate splicing. This is mediated by adenosine deaminases acting on RNA (ADAR) enzymes, that deaminate adenosine bases to produce inosine, which is biochemically read as guanosine. The deaminase domains of ADAR enzymes have now been harnessed and engineered for programmable base editing of specified loci with RNA targeting mechanisms, including CRISPR-Cas13 (**Table 5**).

6.3.1 Cas13-based RNA editing

The first developed CRISPR-RNA base editing system was REPAIR developed by the Feng Zhang group (Abudayyeh et al., 2019; Cox et al., 2017). REPAIR, which stands for RNA editing for Programmable A to I Replacement, was developed using catalytically inactive PspCas13b and hADAR2 deaminase domain (hADAR2$_{DD}$) with the E488Q mutation. Inactivated Cas13b retains its RNA-targeting capacity while catalytic activity is abolished, essentially guiding ADAR enzymes to desired target site for base editing. This proved to efficiently perform Adenosine to Inosine (A → I) base edits. Inosine, during translation, is read as guanosine, effectively producing an A → G edit.

REPAIRx was subsequently developed by inserting hADAR2$_{DD}$ between CasRx. This system was shown to possess superior efficiency and specificity (Liu et al., 2020).

Following REPAIR, the RESCUE (RNA Editing for Specific C-to-U Exchange) system was developed (Abudayyeh et al., 2019). Using directed evolution on the adenine deaminase domain, an engineered domain that was able to act as both an adenine and cytidine deaminase was developed. This was similarly fused to inactive RanCas13b from *Riemerella anatipestifer* to produce the C → U base editing construct (Abudayyeh et al., 2019). Adenosine deaminase activity is retained in RESCUE and thus adenine and cytosine deamination can be multiplexed. Off-target A to I edits can also be prevented through base flips to cause mismatches in the crRNA. This allows RESCUE to function as a highly specific and programmable base editing system to target disease-causing mutations. To improve specificity of the RESCUE system, an additional point mutation (S375A) in ADAR2$_{DD}$ was introduced to develop RESCUE-S, which has significantly lower off-target C to U and A to I edits (Abudayyeh et al., 2019).

Recently, we have shown RNA base editing with Cas13X.1-ADAR2$_{DD}$ *in vitro* by targeting a mutant mCherry gene. The introduction of a stop codon within the mCherry sequence with a G→A mutation abolished mCherry fluorescence when expressed as a plasmid within HEK239FT cells. When the Cas13X.1-ADAR2$_{DD}$ base editor was specified to target the mutated adenosine, we observed ~50% recovery of fluorescence. We also targeted the same mutation using the CRISPR-Cas-inspired RNA targeting system (CIRTS, a protein engineering strategy for constructing programmable RNA control elements) base editor, however this demonstrated a lower recovery of fluorescence (**Figure 7**). The development of these and similar compact Cas13 RNA base editors will greatly facilitate delivery in viral vectors, and these initial *in vitro* results demonstrate the potential for compact RNA editors. We specifically developed and demonstrated single-AAV RNA base editors to ensure the persistent editing that is required for RNA editing therapies, in addition to the safety profile. Further validation of these editors is thus likely to improve the chances of translation of this strategy.

An exclusive cytidine-specific C-to-U editor has also been developed, namely C-to-U RNA editor (CURE). Uniquely, here APOBEC3A was fused to C-terminus of inactive PspCas13b (Huang et al., 2020). The CURE system was compared with RESCUE-S and found to be comparable or greater at on-target efficiencies in the targets tested (*TYMS, ACTB, CTNNB*, *RAS*, *SMARCA4*, and *GAPDH*). When the RESCUE-S system was developed for nuclear localisation (RESCUE-S-N) and compared with CURE in targeting *MALAT1* and *XIST*, CURE clearly outperformed RESCUE-S-N (40% and 28%, respectively vs. 8% and 18% respectively). In terms of off-target effects, the CURE system created similar levels of global off-target edits compared to the RESCUE-S system, but interestingly,

off-target effects made by CURE had a lesser impact on mRNA function. While APOBEC3A was used for base editing, DNA cytidine deamination was not detected from CURE, although this could be attributed to DNA repair mechanisms correcting the edits.

Recently, Tang *et al.* have utilised APOBEC3A for RNA editing specifically. By investigating rationally mutated variants, novel APOBEC3A variants were engineered for RNA-specific activity, through multipoint mutations (Tang et al., 2021a). This is the first time RNA editing activity of APOBEC3A has been retained while abolishing DNA activity, allowing future studies to alternatively incorporate APOBEC3A instead of ADAR with CRISPR-Cas13, for improved specificity and efficiency in C→U editing. The size of APOBEC3A at approximately 200aa is also beneficial for *in vivo* applications.

6.3.2 Other exogenous ADAR RNA base editing systems

Cas13-ADAR editing systems can be defined as exogenous ADAR systems- the $ADAR_{DD}$ is overexpressed by exogenous delivery, rather than relying on harnessing endogenously expressed ADAR in cells. The Cas13-ADAR systems all build on other described RNA editing systems (Fry et al., 2020).

One of the first described RNA editing systems used the bacteriophage-derived λN peptide fused to $ADAR2_{DD}$, which binds short stem-loop RNA structures called BoxBs (Baron-Benhamou et al., 2004; Montiel-Gonzalez et al., 2013; Montiel-González et al., 2016). Although likely less efficient than dPspCas13b-ADAR2(E488Q) (Cox et al., 2017), this minimal system is able to be delivered with AAV, and has been used to edit *Mecp2* in primary murine neurons (Sinnamon et al., 2017; Sinnamon et al., 2020). Off-target events seem to be particularly prevalent with the BoxB-λN-ADAR system, although localizing expression to the nucleus may improve these (Vallecillo-Viejo et al., 2018).

The MS2 bacteriophage coat protein (MCP) editing system fuses $ADAR_{DD}$ to MCP. MCP binds MS2 RNA stem-loop structures attached to gRNAs for site-directed editing (Azad et al., 2019; Katrekar et al., 2019; Tohama et al., 2020). Notably, MCP-$ADAR1_{DD}$(E1008Q) and MCP-$ADAR2_{DD}$(E488Q) constructs have been delivered by AAV *in vivo.* An AAV8-MCP-$ADAR1_{DD}$(E1008Q) construct delivered with an MS2 gRNA demonstrated 2% on-target efficiency and partial restoration of dystrophin expression in an *mdx* mouse model of muscular dystrophy (Katrekar et al., 2019).

The GluR2 system uses the Q/R motif in the *GRIA2* transcript, a natural target for the binding of the dsRBD domains of full-length ADAR2. Attaching an optimised Q/R hairpin motif to gRNA sequences recruits full-length ADAR2 (Katrekar et al., 2019; Wettengel et al., 2017). In addition, the ADAR2 and ADAR2 (E488Q) sequences were delivered with GluR2-gRNAs via AAV8 intramuscularly to the

mdx mouse and systemically to the sparse fur ash (*spf$_{ash}$*) mouse model of ornithine transcarbamylase (OTC) deficiency (Katrekar et al., 2019). This resulted in the low-level correction of a stop codon and splice defect in each model respectively, with resultant rescue of protein expression.

The SNAP-ADAR system uses SNAP-tags, enzymes that form covalent linkages with a O6-benzyl-guanine (BG) substrate (Stafforst and Schneider, 2012). SNAP-tags fused to ADAR$_{DD}$ can bind to gRNAs conjugated with BG that then bind an RNA target (Stafforst and Schneider, 2012; Vogel et al., 2018; Vogel et al., 2014). Although they demonstrate high efficiency and specificity *in vitro*, the gRNAs must be chemically modified for the attachment of the BG and for nuclease-protection and this limits their clinical utility thus far.

Finally, to develop an entirely human-derived editing system to overcome fears of immunogenicity from bacterial derived effectors, the synthetic CRISPR-Cas Inspired RNA Targeting system (CIRTS) was developed. Mimicking Cas13 ssRNA recognition, binding and gRNA targeting functions, a combination of human-derived elements were assembled and fused to ADAR$_{DD}$(E488Q) to develop RNA base editors (Rauch et al., 2019). By identifying the essential elements underlying CRISPR-Cas13 based RNA knockdown, the authors developed an RNA targeting system from human proteins. For example, to perform base editing, the human hairpin-binding domain U1A (TBP6.7) was used as the RNA hairpin binding domain, ADAR2$_{DD}$ was the effector protein and β-defensin 3 was the ssRNA binding domain. Base editing with ADAR2$_{DD}$ (E488Q) led to recovery of the luciferase reporter by approximately 40%. The size of CIRTS base editor also allows for single AAV delivery. While overall efficiency of CIRTS RNA knockdown is lower than CRISPR-Cas13 systems, their small size, versatility, and customisable design are attractive for therapeutic applications.

The authors subsequently developed an inducible RNA base editing system using CIRTS (Rauch et al., 2020). Using the heterodimerization domains (ABI and PYL) of the abscicsic acid (ABA) system fused to CIRTS and ADAR2$_{DD}$ respectively, CIRTS can perform base editing that inducible through administration of ABA.

6.3.3 RNA editing with endogenous ADAR

While exogenous ADAR enzymes delivered with Cas13 are efficient at RNA base editing, there are concerns of immunogenicity, off-target effects and toxicity from overexpression. An alternative strategy is to employ native ADAR enzymes for RNA base editing. LEAPER (leveraging endogenous ADAR for programmable editing of RNA) was developed using engineered RNAs that recruit endogenous ADAR enzymes (arRNAs) for A to I base editing (Qu et al., 2019). Achieving up to 80% editing efficiency and minimal off-target effects in multiple cell types, its application for therapeutics

is yet to be seen. Another recent development is that of RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA editing) which was reported to have almost no off-target editing (Merkle et al., 2019). The therapeutic potential of these platforms against ocular disease remains to be investigated.

In addition to the exogenous SNAP-ADAR systems previously discussed, SNAP-tags can also be attached to adRNAs for recruiting endogenous ADAR. Both ADAR1 and ADAR2 can be recruited by incorporating a SNAP-tag and HALO-tag with arRNAs. In addition, the group further demonstrated recruitment of both ADAR2 and APOBEC1 for concurrent A→I and C→U editing (Stroppel et al., 2021). While SNAP-tag systems can overcome the challenges with ectopic expression of ADAR enzymes, they do not allow delivery with viral vectors due to chemical modifications and overexpression of ADAR enzymes lead to massive off-targets. Recently, CLUSTER gRNAs have been developed that allow to be genetically encoded and delivered using viruses. Here, a cluster of recruitment sequences (10-20nt) are included with the gRNA for binding with mRNA target at various regions. This was shown to increase editing efficiency approximately 30-fold with minimal off-target effects (Reautschnig et al., 2022).

Altogether, for now, it appears the ideal RNA base editing system for clinical application would need to be genetically encoded, delivered via viral vectors with optimal deaminase activity.

6.3.4 Towards clinical development of RNA base editing for ocular gene therapy

RNA base editing approaches using ADAR have been tested on a wide range of disease-causing mutations *in vitro*, but this technology is nascent relative to the plethora of investigations into DNA editing. RNA base-editing mediated by ADAR enzymes has yet to be extensively studied in animal models but has shown promise for therapeutic applications with clinically useful editing rates and improving specificity (Aquino-Jarquin, 2020; Katrekar et al., 2019).

For ophthalmic disorders, inherited retinal diseases are an obvious candidate for therapeutic application, and there are many common mutations that could be edited with an RNA base editing strategy (Fry et al., 2021; Schneider et al., 2021), There are however unfortunately few animal models with G→A or T→C point mutations amenable for RNA editing to test this therapeutic approach (**Table 6**). The development of new animal models will allow testing of these approaches in future.

For translational purposes, it would be ideal to test RNA base editing in human tissues. While the availability of human tissues that carry relevant retinal mutations is unknown, a previous analysis of the Leiden Open Variation Database (LOVD) has revealed the most common pathogenic alleles in IRDs (Fry et al., 2021). A number of these mutations are amenable to RNA base editing, and testing

against them would greatly facilitate clinical translation of this approach. A key limitation of animal models for testing of base editing is that while genes can be highly conserved between species, the nucleotide context surrounding the mutation can be different, and this affects gRNA design and off-target analysis. A research priority therefore, is to select target mutations and develop patient-derived iPSC and retinal organoid lines containing these mutations. Identifying suitable patients through databases and collaborations between research groups will be essential for this. Aligning the concept, we analysed an Australian cohort (n=441) with known genotype and phenotype for editable variants. In this cohort, 33 (8%) patients had mutations that were correctable by current RNA base editors (**Figure 8A and 8B**). As iPSCs have been collected for these patients, RNA base editing can be validated as a therapeutic using patient-derived iPSCs and retinal organoids. A smooth pipeline for the translation of RNA base editing gene therapies can thus be created (**Figure 8C**).

A difficulty with current Cas13-based RNA base editing systems is that their large size prevents delivery within a single AAV. In 2021, the smallest Cas13 enzymes, Cas13X and Cas13Y were reported and employed with ADAR for RNA base editing. Specifically, the Cas13X.1 enzyme was used as it was the smallest amongst the newly identified enzymes. Cas13X.1, with a size of 775aa, could be fused to $ADAR2_{DD}$ at the C-terminal for efficient base editing. To develop a more compact system, the authors truncated Cas13X.1 from both N- and C-terminals to show efficient base editing with a shortened form of Cas13X.1 with a size of 445aa (Xu et al., 2021). Conversion rates were up to 60% for adenine base editors and up to 70% with cytosine base editors, when a range of endogenous genes were targeted. Their efficiencies were also reported to be higher than previously described RNA base editors (Xu et al., 2021). As truncated variants of Cas13X enzymes also retain adequate RNA-targeting ability, more effectors can be identified to explore further functions. Many AAV-compatible RNA editing strategies are now conceivable.

6.4 Future directions and considerations for CRISPR-Cas13

A main reason for the rapid uptake of CRISPR systems by researchers around the world is its ease of application for both *in vitro* and *in vivo* studies. CRISPR enzymes are inexpensive and readily available as the pioneering scientists have made their plasmids available online for everyone. Preclinical studies have also shown extreme promise for its improved efficacy compared to all previous gene-editing and RNA-targeted approaches, explaining the abundance of CRISPR-based gene therapies in preclinical studies and clinical trials (Wu et al., 2020b).

While targeting RNA allows us to address a remarkable number of diseases, achieving effective and safe delivery *in vivo* can limit clinical potential. siRNA drugs for ocular disease have been mired due to unpredictable off-target effects. CRISPR promises a solution through reduced off-target effects, and greater specificity can be achieved by modifying guide RNA length.

As more bioinformatic analyses are conducted, novel CRISPR loci may be uncovered. It was during drafting of this manuscript that the Cas13X, Cas13Y, Cas13bt and Cas13ct enzymes were described (Kannan et al., 2021; Xu et al., 2021). Cas12g has already been found to cleave both ssDNA and ssRNA, blurring the line between conventional DNA-targeting CRISPR and the emerging RNA-targeting CRISPR systems (Smargon et al., 2020). It remains to be seen if more novel RNA-targeting Cas enzymes that triumph existing systems will be identified, however, the various classes of CRISPR systems identified in merely a decade is promising for a myriad of commercial applications.

7 Future perspectives on ocular RNA-targeting therapeutics

7.1 RNA as a therapeutic target

Targeting RNA has a number of advantages for the development of therapeutics (**Table S4**). As the majority of strategies such as ASOs, RNAi and CRISPR-Cas13 interact with their targets via Watson-Crick base-pairing, design of these strategies is relatively straightforward. Once RNA target sequences are known, gRNAs or ASOs can be rationally designed, with potential off-target sites predicted and reduced. This also allows for the custom design of therapeutics to target patient specific sequences, or specific alleles.

In contrast, identification of drugs targeting protein often require large scale screening methods of small molecules, medicinal chemistry optimisation and complex production methods. Furthermore, targeting RNA allows targeting of ‘undruggable’ protein targets such as non-coding RNAs or proteins with conformations limiting small molecule binding.

An additional clinical appeal of targeting the transcriptome is that unlike editing the genome, edits are not permanent. This is pertinent considering our increasing understanding of off-target effects. When off-target edits occur in DNA, the resulting mutation may have permanent undesired consequences. This greatly reduces clinical applicability of such methods. Off-target edits in the RNA, however, are of relatively lower risk due to transient nature of RNA making mutations readily reversible if the system can be turned off or requires redosing, though it is important to note that RNA off-target effects in an oncogene may be detrimental (Teoh et al., 2018).

Conditions in which only temporary therapeutic effect is required are ideal for RNA-targeted therapeutics, as modifying DNA leaves a permanent change even after therapeutic effect has been achieved. This also means RNA editing can be used for non-genetic diseases for which DNA editing may be inappropriate or unsafe (Liu et al., 2020). RNA also plays a myriad of functions other than coding for proteins, in the form of ncRNAs; many of which have been implicated in ocular diseases (Song and Kim, 2021). This widens the range of targets for therapeutic development. An interesting example is that of Alu RNA. This retrotransposon element is reverse transcribed and integrated into the genome. However, Alu can also be reverse transcribed into cDNA, leading to RPE toxicity in mice. Inhibition of the reverse transcription using nucleoside RT inhibitors is being studied as a potential therapeutic (Fukuda et al., 2021).

Finally, relative to DNA targeting, the cytoplasmic localisation of RNA makes it easier to target RNA without the need for delivering therapeutic components to the nucleus. As this article aims to review RNA-targeted therapeutic strategies, specific details of RNA-targeted therapeutics in preclinical studies are beyond the scope of this review. They are, however, available in the supplementary tables to provide an overview of the field's progress.

7.2 Alternative targets to RNA

While the developments with ASO, RNAi and CRISPR-Cas13 have showcased the advantages of targeting disease at the RNA level, RNA is a relatively nascent therapeutic target, compared to years of drug development targeting proteins, and more recently, DNA.

Currently, in targeting protein, monoclonal antibodies are leading the field and have become the predominant and best-selling drugs in the pharmaceutical market. Seventy-nine therapeutic antibodies have thus far been approved by the US FDA, with 18 of the approved just after 2018 (Lu et al., 2020). For ocular disease, several monoclonal antibodies have been approved for treatment of neovascularisation. They are namely, bevacizumab, ranibizumab and brolucizumab, all of them targeting the VEGF-A protein. Aptamers like Pegaptinib and recombinant fusion proteins like Aflibercept targeting VEGF-A protein have also been approved (Lin et al., 2020). While effective, their efficacy is short-lived, requiring frequent injections that cause discomfort and anxiety, increases risk of complications such as submacular haemorrhage and retinal detachment, can be toxic to the eye and does not necessarily prevent recurrences (Garweg et al., 2021; Lin et al., 2020). Furthermore, regular treatment is a burden financially and logistically for both patients and healthcare providers.

DNA editing therapeutic options include programmable nucleases like zinc finger nucleases (ZFNs) and transcription activator-like endonucleases (TALENs), and now DNA targeting with CRISPR-Cas. ZFN and TALEN-based gene therapy require engineering of new enzymes for each new DNA target, and widespread adoption of these gene editing technologies for clinical applications was hampered by the relative complexity of their design and difficulties in delivery for *in vivo* editing. CRISPR transformed this field by making both design and execution simpler. A plethora of technologies for DNA editing have now been developed using CRISPR technology (for comprehensive review see (Anzalone et al., 2020). All DNA targeting strategies offer the advantage of creating a single DNA edit that could result in permanent treatment. While highly attractive, as discussed this carries the corresponding risk of creating permanent DNA edits in unintended loci that cannot be reversed.

7.3 Off-target effects with RNA-targeted therapeutics

Like other therapeutics, off-target effects occur with RNA targeting therapies. These can be characterised as 1) hybridisation dependent, due to unintended hybridisation to a similar target sequence and 2) hybridisation independent effects due to non-Watson-Crick interactions with other RNA sequences or proteins (Frazier, 2014).

Off-target effects have been well-recognised in ASO and siRNA therapeutics (Watts and Corey, 2012). Both have the potential to bind to non-target transcripts with partial complementarity. In the case of siRNAs, as little as 8nt of complementarity between an siRNA and a 3' UTR of an unintended mRNA sequence, can lead to change of gene expression up to 4-fold, in up to hundreds of genes (Birmingham et al., 2006). Even when ASO or siRNA is not specific for cellular RNA, significant transcriptome-wide changes have also been observed (Stojic et al., 2018).

It remains to be seen how well CRISPR-Cas systems targeting RNA can be engineered to limit off-target effects, as they have been for DNA-targeting CRISPR systems. From early studies, no off-target effects were observed with CRISPR-CasRx as compared to shRNA with over 900 off-target effects when endogenous annexin A4 (*ANXA4*) was targeted in mammalian cells (Konermann et al., 2018). However, recent studies have shown similar levels of toxicity and off-target effects for CRISPR-Cas13 systems compared to shRNA. In this study, CasRx notably exhibited low off-target effects in HEK293FT cells (Özcan et al., 2021). More recent analyses have shown that off-target activity of CasRx is dependent on target gene, and that CasRx collateral activity of bystander RNAs can be significant with abundant genes such as ferritin heavy chain 1 (*FTH1*) and (Heterogeneous Nuclear Ribonucleoprotein A2/B1 (*HNRNP-A2B1*) (Shi et al., 2021). To address this concern, Tong and colleagues have recently reported the development of CasRx and Cas13X variants, namely high fidelity CasRx (hfCas13d) and Cas13X (hfCas13X) derived through mutagenesis (Tong et al., 2021b). Both hfCas13d and hfCas13X could function efficiently on par with their wildtype strains, and cell growth remained unaffected. Importantly, collateral effects were markedly reduced or almost eliminated. Other studies have also shown that guide RNA length can be manipulated to greatly improve specificity, and with bioinformatic tools available to predict off-target sites, they can almost completely be avoided (Labun et al., 2019). Higher fidelity nucleases are also expected to be identified with more bioinformatic analyses (Epstein et al., 2021).

Nevertheless, off-target effects remain a concern for RNA base editing with ADAR enzymes. The commonly used hyperactive ADAR2$_{DD}$-E488Q mutation produces high efficiencies, however this is known to increase off-target editing throughout the transcriptome (Cox et al., 2017; Vallecillo-Viejo

et al., 2018). Further engineering has produced enzyme mutants such as the ADAR2$_{DD}$-E488Q/T375G double mutant, which shows greater specificity although with some loss of on-target efficiency (Cox et al., 2017). Strategies such as using high specificity ADAR mutants (Abudayyeh et al., 2019; Cox et al., 2017), localization of editing to the nucleus(Katrekar et al., 2019; Vallecillo-Viejo et al., 2018), and design of guide RNAs to install mismatched guanosines at common editing sites within the gRNA binding region (Qu et al., 2019) all represent advances that can improve off-target rates.

7.4 Delivery of RNA-targeted therapeutics

A major consideration when developing RNA-targeting ocular gene therapies is delivery to the eye. It is important to note that systemic administration for ocular therapy is not feasible owing to the blood-ocular barrier and therefore local administration is necessary (Yu and Wu, 2020). For delivery to the anterior eye, topical, subconjunctival or intracameral administration is generally used. For the posterior eye, intravitreal, subretinal or suprachoroidal administration is performed. Retinal diseases typically require delivery to the posterior eye, and subretinal administration is commonly used in clinical settings as the drug is delivered directly between the photoreceptors and RPE cells. For example, Luxturna is delivered subretinally to target the RPE cells (High and Roncarolo, 2019). While subretinal administration provides clinical benefit, the method is highly invasive and requires prior vitrectomy to be performed. The less invasive intravitreal injection is primarily used for delivery to the outer retina (e.g., retinal ganglion cells), as retinal transduction is poorer due to the vitreous and inner limiting membrane. Recently, suprachoroidal delivery has also a shown similar transduction profile to subretinal delivery of the outer retina, while being less invasive (Ding et al., 2019; Yu and Wu, 2020).

7.5 Commercial landscape of RNA-targeted therapeutics for ocular conditions

The field of gene therapy is currently a focus for much commercial activity (Garafalo et al., 2020). Since 2016, commercial sponsors have become dominant figures in Investigational New Drug (IND) applications, revealing an enthusiasm among biopharmaceutical companies in commercialising gene therapies. Currently, there are no approved ASO, siRNA or CRISPR-Cas therapy for any ocular condition on the market, however RNA-targeting drugs are an emerging area of biopharmaceutical interest with several corporates such as Ionis Pharmaceuticals, Quark Pharmaceuticals, Ribometrix, PYC Therapeutics and Arrakis Therapeutics, have been set up for the development of RNA-targeting drugs (Thavarajah et al., 2021). Currently, clinical trials predominantly focus on inherited retinal degeneration including those associated with the genes *CEP290*, *RHO* and *USH2A*.

The most advanced of these candidates appears to be the intravitreal ASO QR-110 (ProQR Therapeutics, Netherlands) to treat the CEP290 c.2991+1655A>G allele. Encouraging early results (NCT03140969) suggest a recovery of visual function in some patients (Cideciyan et al., 2019).

Ionis Pharmaceuticals, a leader in antisense drugs and the developer of four out of the nine approved ASO drugs to date, currently has 40 RNA-targeted therapeutics in development and 4 drugs are in Phase III trials. Ionis Pharmaceuticals is now developing two ASO drugs for ocular disease, namely IONIS-FB-L_{Rx} for geographic atrophy due to AMD and ION357 (or QR-1123, now licensed by ProQR Therapeutics) for adRP. Both candidates are currently in Phase II studies. Quark Pharmaceuticals has a drug in the pipeline for targeting ischaemic optic neuropathy and open angle glaucoma, known as QPI-1007. Clinical studies targeting ischaemic optic neuropathy has progressed to phase III trials. PYC Therapeutics has developed a drug to modulate CNOT3 expression to treatment another common form of adRP, RP11(Grainok et al., 2021).

For RNAi therapeutics, Alnylam Pharmaceuticals is the current leader, having produced all three approved siRNA drugs to date. No siRNA drugs for ocular disease are on their pipeline.

Shape Therapeutics is investigating the recruitment of endogenous ADAR RNA editing and suppression of premature stop codons to address ocular disease. In their pipeline are gene therapies to address Stargardt disease, retinitis pigmentosa, Usher syndrome and AMD. Preclinical studies for these, however, are not available.

While no RNA targeting CRISPR-therapeutics are in clinical trials, Editas Medicine, unlike other CRISPR pharmaceutical companies, is focusing exclusively on ocular diseases. Results are awaited from phase I/II trials (NCT03872479) of EDIT-101 (Editas Medicine, USA) for treatment of the same *CEP290* c.2991+1655A>G mutation. This AAV5-delivered DNA targeting CRISPR-based therapeutic uses SaCas9 to target the *CEP290* gene and remove an intronic mutation from the genome with two gRNAs to genomic region flanking the intronic mutation. Interestingly, this trial marked the first-in-human CRISPR gene therapy when the drug was administered to the first patient. Two other drugs for Usher syndrome (EDIT-102) and retinitis pigmentosa 4 are also in the pipeline for Editas Medicine, however clinical trials have yet to commence.

8 Future directions and conclusions

Currently, gene editing technology is being developed to treat fatal or debilitating diseases in both adults and children (McCaughey et al., 2016; Wang et al., 2017), and many applications in the eye are

conceivable. In light of potential permanent off-target effects introduced by DNA targeting systems, how do we proceed without compromising safety? Targeting RNA may be a solution with the range of efficient and precise RNA-targeting systems now available (Damase et al., 2021).

Going forward, we predict that development of gene therapies against many ocular diseases will increase dramatically in the next few years with anti-VEGF therapy predicted to be one of the next approved gene therapies (Guimaraes et al., 2021). We and others have shown the feasibility of RNA editing for *VEGF* knockdown as a potential therapeutic for ocular neovascular disease (Chuang et al., 2021b; Zhou et al., 2020a). If successful, RNA silencing of VEGF may prove to be superior alternatives to current anti-VEGF drugs that require frequent intraocular injections. Several other genes may also be targeted similarly for treatment of common ocular disease.

In addition, inherited retinal disease have been a key focus of many RNA targeting therapeutics thus far. Our preliminary work with Cas13X.1-ADAR2$_{DD}$ RNA base editors demonstrate the *in vitro* efficacy of compact base editors, with future work planned to deliver these within single AAV systems with a goal to achieve safe and efficient RNA base editing. Alternatively, engineered guide sequences have also been described for RNA base editing through endogenous ADAR enzymes, possessing lower rates of off-target editing, although efficiency is compromised. The validation of these tools in upcoming animal studies will showcase the potential of clinical RNA base editing.

Beyond inherited retinal disease, the big four ocular diseases responsible for the greatest burden of blindness are cataracts, diabetic retinopathy, AMD and glaucoma (Heath Jeffery et al., 2021; Schmidt et al., 2021). The incidence of these conditions is projected to significantly increase by 2050. Other than cataracts, gene therapy options for these conditions have been extensively studied in preclinical and clinical studies (Komáromy et al., 2021; Lin et al., 2020). Given the prevalence of these conditions, developing gene therapies for their treatment would have major cost implications for health systems. There is significant potential for RNA targeting therapeutics to address modifiable pathways in these diseases.

This review has demonstrated a wide range of RNA-targeting therapeutics that have potential for treating ocular disease. ASO and siRNA therapeutics have a long history of development and a number of ASO therapeutics against IRDs, primarily from ProQR Therapeutics, have shown promise in clinical trials. These are expected to further our understanding on the beneficial effect of targeting RNA for ocular gene therapy (Xue and Maclaren, 2020) and further encourage research into the field, as the approval of Luxturna® did previously.

The most exciting emerging aspect of RNA therapeutics is now using gene editing tools such as CRISPR-Cas. CRISPR-Cas13 systems in particular, are a valuable addition to the RNA therapeutics toolbox. As a versatile platform with RNA knockdown, multiplexed RNA-targeting, inducible RNA-targeting, and RNA base editing capabilities, Cas13 enzymes may prove to be powerful agents against both common and inherited ocular diseases. The rapid rate of progress in CRISPR research is evident from the plethora of gene therapies currently in clinical trials within a decade of the technology's first description. As the potential of Cas13 systems is realized widely in the research community and is taken up for developing novel therapies, its utility against ocular diseases will be probed further. This is expected due to the advantages of targeting the eye, and ease of monitoring without invasive methods (Xu et al., 2018).

The approval of Patisiran (Onpattro®), the first RNAi drug, came two decades after discovery of the gene silencing strategy (Adams et al., 2018). Luxturna® was FDA approved a decade after initial application. There is hope that future approvals may not take decades as researchers and regulators venture together into this brave new world. RNA, that started off life on earth and brought it to a standstill with the recent pandemic, is also now showing us a way to cure many major diseases that affect mankind.

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Tables

Table 1. List of approved ASO and RNAi drugs. Other than Fomivirsen, all approved ASO and RNAi drugs are still available in the market.

Table 2. Clinical trials completed or currently underway for ASO-based ocular therapeutics.

Table 3. Clinical trials of siRNA-mediated therapies for ocular disease.

Table 4: Common ocular conditions and their causative genes that have been targeted for gene silencing in clinical and preclinical studies. These genes are ideal candidates for CRISPR-Cas13 based gene knockdown therapies. Detailed outcomes of these studies can be found in **Tables S1 and Table S2**.

Table 5. RNA base editors developed to date.

Table 6. Summary of the current IRD mouse models with G>A or T>C transitions listed in the Mouse Genome Informatics (MGI) database adapted from Collin et al. to Prevalence of families affected in the UK sourced from Pontikos et al.

1771 **Table 1.** List of approved ASO and RNAi drugs. Other than Fomivirsen, all approved ASO and RNAi drugs are still available in the market.

Strategy	Drug	Disease	Delivery	Sponsor	Status	References
ASO	Fomivirsen	Cytomegalovirus Retinitis	Intravitreal	Ionis Pharmaceuticals	Approved by US FDA in 1998, EMA in 1999. Withdrawn from the market.	(Roehr, 1998)
	Mipomersen	Homozygous Familial Hypercholesterolemia	Subcutaneous	Ionis Pharmaceuticals/Sanofi Genzyme	Approved by US FDA in 2013, Currently available.	(Wong and Goldberg, 2014)
	Eteplirsen	Duchenne Muscular Dystrophy	Intravenous	Sarepta Therapeutics	Approved by US FDA in 2016 Unauthorised by EMA. Currently available.	(Lim et al., 2017)
	Nusinersen	Spinal muscular Atrophy	Intrathecal	Ionis Pharmaceuticals/ Biogen	Approved by FDA in 2016, EMA in 2017. Currently available.	(Goodkey et al., 2018)
	Inotersen	Hereditary transthyretin-mediated amyloidosis	Subcutaneous	Akcea Therapeutics and Ionis Pharmaceuticals	Approved by FDA and EMA in 2018. Currently available.	(Mathew and Wang, 2019)
	Golodirsen	Duchenne Muscular Dystrophy	Intravenous	Sarepta Therapeutics	Approved by FDA in 2019. Currently available.	(Heo, 2020)
	Volanesorsen	Familial chylomicronaemia syndrome	Subcutaneous	Akcea Therapeutics	Approved by EMA in 2019. Currently available.	(Esan and Wierzbicki, 2020)
	Viltolarsen	Duchenne Muscular Dystrophy	Intravenous	NS Pharma	Approved by FDA in 2020. Currently available.	(Dhillon, 2020)
	Casimersen	Duchenne Muscular Dystrophy	Intravenous	Sarepta Therapeutics	Approved by FDA in 2021. Currently available.	(Shirley, 2021)
RNAi	Patisiran	Hereditary transthyretin-mediated amyloidosis	Intravenous	Alnylam Pharmaceuticals	Approved by FDA in 2018. Currently available.	(Hoy, 2018)

	Givosiran	Hepatic Porphyria	Subcutaneous	Alnylam Pharmaceuticals	Approved by FDA in 2019. Currently available.	(Scott, 2020)
	Lumasiran	Primary hyperoxaluria	Subcutaneous	Alnylam Pharmaceuticals	Approved by FDA and EMA in 2020. Currently available.	(Scott and Keam, 2021)

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1773 **Table 2.** Clinical trials completed or currently underway for ASO-based ocular therapeutics.

Disease	**Delivery**	**Mechanism**	**Drug**	**Phase**	**Company**	**NCT number**	**Outcome**	**Reference (other than clinicaltrails.gov)**
Cytomegalovirus retinitis	Intravitreal	Binds to major immediate-early transcription unit, Blocks viral replication	Fomivirsen sodium	Approved by US FDA in 1998, Withdrawn.	Ionis Pharmaceuticals, Inc.	NCT00002187	Two dose schedules administered to patients with AIDS-related Cytomegalovirus retinitis. Treatment delayed disease progression and was well tolerated with no significant complications.	(Henahan, 1998)
Retinitis pigmentosa, Usher syndrome Type 2	Intravitreal	Target *USH2A* exon 13 mutation by exon skipping	QR-421a	Phase I/II Recruiting	ProQR Therapeutics	NCT03780257	24 months follow up study after single intravitreal injection of three different doses (50, 100, or 200 µg) in 18 RP patients with *USH2A* exon 13 mutation. Follow up will be for 24 months to study safety and effect on visual function. Interim results at 3 months show visual improvement with low and middle doses, and no serious adverse effects have been reported.	(Xue and Maclaren, 2020)
Retinitis pigmentosa, Usher syndrome Type 2	Intravitreal	Target *USH2A* exon 13 mutation by exon skipping	QR-421a	Phase II/III Recruiting	ProQR Therapeutics	NCT05158296	2 dose levels (180µg and 60µg) to be administered with maintenance doses (60µg) at 3 months and 6 months. Patients will be monitored for at least 18 months and benefit-risk will be assessed against sham procedure.	
Retinitis pigmentosa,	Intravitreal	Target *USH2A* exon 13 mutation by exon skipping	QR-421a	Phase II/III Active, not recruiting	ProQR Therapeutics	NCT05176717	2 dose levels (180µg and 60µg) to be administered with maintenance doses (60µg) at 3 months and 6 months. Mean sensitivity will be	

Usher syndrome Type 2							assessed against sham procedure 12 months from administration.	
Geographic atrophy	Subcutaneous	Target *CFB*	IONIS-FB-L_{Rx}	Phase II Recruiting	Ionis Pharmaceuticals, Inc.	NCT03815825	330 patients tested for fundus autofluorescence after treatment with 3 varying doses. Phase I studies showed up to 72% plasma FB reduction with multiple injections. No adverse effects were also observed.	(Jaffe et al., 2020)
Geographic atrophy	Intravitreal	Target *C5*	Zimura	Phase II/III Completed	IVERIC bio, Inc.	NCT02686658	27.4% reduction in mean Geographic atrophy growth rate over 12 months. No drug-related toxicity, severe side effects or endophthalmitis were observed after 12 months indicating good tolerance of drug.	(Jaffe et al., 2021)
Leber's congenital amaurosis	Intravitreal	Target c.2991+1655A>G Mutation in Centrosomal *CEP290*	QR-110 (sepofarsen)	Phase I/II Completed	ProQR Therapeutics	NCT03140969	3 dose levels tested in 11 participants, with a maximum of 4 doses over 1 year. No adverse effects were reported, and vision improved after 3 months. Progressed to Phase II/III trials.	(Cideciyan et al., 2019; Koulisis and Nagiel, 2020)
Leber's congenital amaurosis	Intravitreal	Target c.2991+1655A>G Mutation in *CEP290*	QR-110 (sepofarsen)	Phase I/ II, active, not recruiting	ProQR Therapeutics	NCT03913130	First dose of sepofarsen to be followed by maintenance dose every six months for 24 months to study long term safety, tolerability, and efficacy. No study results posted.	
Leber's congenital amaurosis	Intravitreal	Target c.2991+1655A>G Mutation in *CEP290*	QR-110 (sepofarsen)	Phase II/ III, Active, not recruiting	ProQR Therapeutics	NCT03913143	Low dose and high dose of sepofarsen compared with sham control in 36 patients. Maintenance dose at 3 months and every 6 months thereafter for 24 months is to be given. No study results posted.	(Xue and Maclaren, 2020)

Leber's congenital amaurosis	Intravitreal	Target c.2991+1655A>G Mutation in *CEP290* in children (<8 years)	QR-110 (sepofarsen)	Phase II/III Recruiting	ProQR Therapeutics	NCT04855045	Dose escalation study, followed by randomized study with 2 dose levels in 15 participants over 24 months. No study results posted.	
Ischaemic central retinal vein occlusion, neovascular glaucoma	Topical eye drops	Target *IRS-1*	Aganirsen	Phase II/III Unknown status	Gene Signal SAS	NCT02947867	Two dose levels tested in 333 participants for 24 weeks. Study status is unknown. A related study evaluated 69 patients and has shown 26.2% reduction of corneal neovascularisation area after 90 days, which lasted until 180 days. Adverse events were also lower compared to placebo.	(Cursiefen et al., 2014)
Autosomal dominant retinitis pigmentosa	Intravitreal	Target mutant *RHO* mRNA and preserve wildtype RHO protein	QR-1123	Phase I/II Recruiting	ProQR Therapeutics	NCT04123626	Single dose and repeat dose studies in 35 participants every trimester over 1 year. Dose related safety and effects on visual function to be studied. No study results posted.	
Primary open angle glaucoma	Intravitreal	Target *TGF-β2* for improving glaucoma surgery outcome	ISTH0036	Phase I, Completed	Isarna Therapeutics GmbH	NCT02406833	12 participants were administered single intravitreal injection at different doses at the end of glaucoma surgery. No drug related adverse effects or toxicities were observed. IOP levels remained low for up to 3 months with high doses of ISTH0036.	(Pfeiffer et al., 2017)

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1776 **Table 3.** Clinical trials of siRNA-mediated therapies for ocular disease.

Disease	**Delivery**	**Target**	**Drug**	**Phase**	**Company**	**NCT number**	**Outcome**	**References (other than clinicaltrails.gov)**
Neovascular age-related macular degeneration	Intravitreal	*VEGFR1*	AGN211745	Phase I/II Completed	Allergan, Sirna Therapeutics Inc.	NCT00363714	Intravitreal injection of different dosages performed in 26 participants. Participants were observed over 24 months. No study results posted. Preclinical studies showed 57% reduction of *VEGFR-1* mRNA levels and 45-66% decrease in neovascularization area.	(Kaiser et al., 2010; Shen et al., 2006)
Neovascular age-related macular Degeneration	Intravitreal	*VEGFR1*	AGN211745	Phase II, terminated	Allergan	NCT00395057	Different doses of AGN211745 given to 138 participants over 2 months, and then observed for 24 months. Company decided to terminate study early. Incomplete study results only available.	
Dry eye disease	Topical eye drops	*TRPV1*	SYL1001	Phase I, Completed	Sylentis, S.A.	NCT01438281	30 participants were tested for tolerance of SYL1001 with two different doses. 6 subjects were initially evaluated for safety with single dose. 24 subjects were treated with multiple ascending doses. Local tolerance was reported as excellent with no serious adverse effects or modifications of ocular surface or iris.	(Gonzalez et al., 2012)
Dry eye disease	Topical eye drops	*TRPV1*	SYL1001	Phase I/II, completed	Sylentis, S.A.	NCT01776658	156 participants were treated with different doses of SYL1001. Treatment with 1.125% SYL1001 once daily led to significant drop in visual analogue scale scores starting from day 4. The same treatment also significantly improved conjunctival hyperemia. Ocular surface disease index questionnaire scores were	(Benitez-Del-Castillo et al., 2016)

							significantly reduced with all doses. Excellent tolerability and no adverse effects were reported.	
Dry eye disease	Topical eye drops	*TRPV1*	Tivansiran/ SYL1001	Phase III, completed	Sylentis, S.A.	NCT03108664	330 participants were treated with one drop of SYL1001 in affected eye. Improvement in all areas of visual analogue scale such as pain, dryness, burning/stinging, itching, foreign body sensation and quality of life after 28 days treatment.	(Gonzalez et al., 2020)
Dry eye disease	Topical eye drops	*TRPV1*	Tivansiran/ SYL1001	Phase III, Recruiting	Sylentis, S.A.	NCT04819269	200 participants with dry eye disease symptoms will be dosed daily for three months to examine efficacy and safety of Tivanisiran.	
Neovascular age-related macular degeneration	Topical eye drops	*NRARP*	SYL18001	Phase I, Recruiting	Sylentis, S.A.	NCT04782271	36 participants to be treated with different doses of SYL1801 to assess safety and tolerability in healthy volunteers. Preclinical studies showed high safety profile and *NRARP* silencing in endothelial cells, resulting in reduced proliferation and migration in rats.	(Jimenez et al., 2019)
Open angle glaucoma	Topical eye drops	*ADRB2*	SYL040012	Phase I/II, Completed	Sylentis, S.A.	NCT01227291	30 participants were administered single dose of SYL040012 daily for 7 days and monitored for 11 days total to assess tolerance and effect. No study results posted.	
Open angle glaucoma	Topical eye drops	*ADRB2*	SYL040012	Phase II, Completed	Sylentis, S.A.	NCT01739244	89 participants subjected to different doses of SYL040012 eye drops for 14 consecutive days to investigate tolerability and intraocular pressure reducing effect. 300 µg/eye/day of SYL040012 resulted in significant reduction of intraocular pressure on day 14. Adverse effects were reported in 14.6% of participants, with most of them mild.	(Gonzalez et al., 2014)

Open angle glaucoma	Topical eye drops	*ADRB2*	SYL040012	Phase IIB, completed	Sylentis, S.A.	NCT02250612	184 participants were 1 drop of SYL040012 of different concentrations and safety and hypotensive effect was monitored after 28 days. No significant difference between the different doses (0.375%, 0.75%, 1.125% and 1.5%) were found, but 0.75% bamosiran showed highest reduction in IOP at day 28. No severe adverse effects were observed.	(Gonzalez et al., 2016)
Glaucoma, ocular hypertension	Topical eye drops	*ADRB2*	SYL040012	Phase I, completed	Sylentis, S.A.	NCT00990743	30 participants were administered SYL040012 as eye drops. 6 subjects were given single dose to evaluate safety. 24 subjects were given daily injections of ascending doses for 7 days. Excellent tolerance to drug was observed, and no adverse effects were noticed. Up to 15% reduction of intraocular pressure was observed on day 4.	(Ruz et al., 2011)
Neovascular age-related macular degeneration, diabetic Retinopathy, diabetic macular edema	Intravitreal	*RTP801*	PF-04523655	Phase II, Completed	Quark Pharmaceuticals	NCT01445899	24 participants were administered PF-04523655 once, with various doses to determine maximum tolerated dose. 240 participants were administered PF-04523655 biannaully to determine efficacy and safety alone and with ranibizumab. No study results posted.	
Diabetic macular edema	Intravitreal	*RTP801*	PF-04523655	Phase II, terminated	Quark Pharmaceuticals	NCT00701181	184 patients were treated with three different doses PF-04523655 every four weeks for six months, then treatment was given as needed. Visual acuity was improved with all doses till 12 months. The 3mg PF-04523655 treatment group showed highest improvement. Drug was safe and well tolerated with minimal adverse effects.	(Nguyen et al., 2012)

Neovascular age-related macular degeneration	Intravitreal	*RTP801*	PF-04523655	Phase I, completed	Quark Pharmaceuticals/ Pfizer	NCT00725686	13 participants were administered single intravitreal injection of escalating doses. Two weeks after treatment, most (80%) of patients showed improved visual acuity, with mean improvement at +8 letters. Remarkably, at day 14, one patient had improvement of 40 letters. No adverse effects were observed.	(Nguyen et al., 2009)
Neovascular age-related macular degeneration	Intravitreal	*RTP801*	PF-04523655	Phase II, completed	Quark Pharmaceuticals/ Pfizer	NCT00713518	152 participants were intravitreally administered two different doses (1mg or 3mg) of PF-04523655 with ranibizumab. Participants were monitored for 4 months for visual acuity, adverse effects, and retinal lesion thickness. No study results posted.	
Diabetic macular edema	Intravitreal	*VEGF*	bevasiranib	Phase II, Completed	OPKO Health, Inc.	NCT00306904	48 patients were subjected to three intravitreal injections with escalating dose every month. Results were obtained four weeks post final injection. Retinal thickness and visual acuity had no significant change immediately after treatment, retinal thickness was significantly decreased after 4-8 weeks.	(Prenner and Group, 2007)
Neovascular age-related macular degeneration	Intravitreal	*VEGF*	bevasiranib	Phase I, completed	OPKO Health, Inc.	NCT00722384	15 participants were intravitreally administered escalating doses of bevasiranib to evaluate tolerability, adverse effects, visual acuity, intraocular pressure and lens opafication. No study results posted.	
Neovascular age-related macular degeneration	Intravitreal	*VEGF*	bevasiranib	Phase III, Terminated	OPKO Health, Inc.	NCT00499590	338 participants received bevasiranib every 8 or 12 weeks, after pre-treatment of 3 doses with ranibizumab, 12 weeks earlier. Safety and efficacy in terms visual acuity was evaluated. Serious adverse effects, like decreased visual acuity and endophthalmitis, were observed with bevasiranib in	

							both frequencies of dose administration for at least 25% of subjects. Study was terminated early.	
Neovascular age-related macular degeneration	Intravitreal	*VEGF*	bevasiranib	Phase II, completed	OPKO Health, Inc.	NCT00259753	120 participants were intravitreally administered different doses of bevasiranib and monitored for 12 weeks for macular edema and visual acuity. No study results posted.	
Neovascular age-related macular degeneration	Intravitreal	*VEGF*	bevasiranib	Phase III, study never initiated	OPKO Health, Inc.	NCT00557791	No study results available.	
Ischemic optic neuropathy	Intravitreal	*CASP2*	QPI-1007	Phase I, completed	Quark Pharmaceuticals	NCT01064505	48 participants were treated with QPI-1007 with single injection of various doses to evaluate safety, tolerability and dose-limiting toxicity over 12 months. no study results posted.	
Ischemic optic neuropathy	Intravitreal	*CASP2*	QPI-1007	Phase II/III. Terminated	Quark Pharmaceuticals	NCT02341560	732 participants were intravitreally administered two different doses of QPI-1007. They were to be monitored for 12 months for visual acuity, safety, and tolerability. Study was terminated with no results posted.	(Solano et al., 2014)
Angle-closure, primary and acute glaucoma	Intravitreal	*CASP2*	QPI-1007	Phase II, completed	Quark Pharmaceuticals	NCT01965106	46 participants were administered single intravitreal dose of 1.5mg QPI-1007 and monitored for up to 6 months for safety, tolerability, and visual acuity. No study results posted.	

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Table 4. Common ocular conditions and their causative genes that have been targeted for gene silencing in clinical and preclinical studies. These genes are ideal candidates for CRISPR-Cas13 based gene knockdown therapies. Detailed outcomes of these studies can be found in **Table S1 and Table S2**.

Ocular condition	Gene	References
Clinical trials		
Geographic atrophy	*CFB*	(Jaffe et al., 2020)
	CF5	(Jaffe et al., 2021)
Glaucoma	*ADRB2*	(Gonzalez et al., 2016; Gonzalez et al., 2014; Ruz et al., 2011)
	IRS-1	(Cursiefen et al., 2014)
	TGF-β2	(Pfeiffer et al., 2017)
Ischemic optic neuropathy	*CASP2*	(Solano et al., 2014)
Dry eye disease	*TRPV1*	(Benitez-Del-Castillo et al., 2016; Gonzalez et al., 2012; Gonzalez et al., 2020)
Neovascular age-related macular degeneration	*VEGFR1*	(Kaiser et al., 2010; Shen et al., 2006)
	RTP801	(Nguyen et al., 2009; Nguyen et al., 2012; Prenner and Group, 2007)
	VEGF	-
	NRARP	(Jimenez et al., 2019)
Preclinical studies		
Diabetic retinopathy	*VEGFA*	(Chuang et al., 2021b; Garrett et al., 2001; Holmgaard et al., 2017; Holmgaard et al., 2020; Lai et al., 2002; Wang et al., 2020c)
	PDGF-B	(Jo et al., 2006)
	VEGFR1, VEGFR2	(Connor et al., 2009; Hagigit et al., 2012; Shen et al., 2006)
	GLUT1	(You et al., 2017)
	PIGF	(Araújo et al., 2020)
Neovascular age-related macular degeneration	*HIF-α*	(Kim et al., 2017; Koo et al., 2018)
	VEGFA	(Ryoo et al., 2017; Zhou et al., 2020a)
	IRS-1	(Cloutier et al., 2012)
Iris neovascularisation	*VEGF*	(Bhisitkul et al., 2005)
Corneal neovascularisation	*VEGFA*	(Murata et al., 2006; Zuo et al., 2010)
	HIF-1α	(Chen et al., 2012)
Glaucoma	*AQP1*	(Wu et al., 2020a)
	MYOC	(Jain et al., 2017)
	NEFL, PVALB, RHO, GRIN1, OPN1SW	(Taniguchi et al., 2020)
	ADRB2	(Martínez et al., 2014)
	CASP2	(Ahmed et al., 2011; Solano et al., 2014)

Optic neuropathy	*DDIT3, SARM1*	(Wang et al., 2020b)
	OPA1	(Bonifert et al., 2016)

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1783 **Table 5.** RNA base editors developed to date.

Base editor	Effector	Binding domain	Application	Outcome	Reference
(λ)N-ADAR	$ADAR2_{DD}$	λN peptide	λN peptide fused to $ADAR2_{DD}$ to recognise boxB hairpins in RNA.	20% correction in *CFTR* and *EGFP* mRNA, in Xenopus oocytes and HEK293T cells respectively, was observed with no off-target editing reported.	(Montiel-Gonzalez et al., 2013)
SNAP-ADAR	$ADAR1_{DD}$	SNAP-tag	ADAR1DD fused to C-terminal of SNAP-tag	60-90% conversion rate with no off-target mutations in the open reading frame.	(Stafforst and Schneider, 2012)
ASO-ADAR	Endogenous ADAR1/2	ASO	Recruiting endogenous ADAR for base editing	75-85% editing efficiency with ADAR1 p150.	(Merkle et al., 2019)
REPAIR	$ADAR2_{DD}$	Cas13b	$ADAR2_{DD}$ fused to C-terminal of inactive Cas13b	Up to 40% editing efficiency achieved targeting endogenous genes for A to I editing. No PFS requirements were required for efficient editing.	(Cox et al., 2017)
RESCUE	$ADAR2_{DD}$	Cas13b	Direct evolution of $ADAR2_{DD}$ to for cytosine deaminase, fused to C-terminal of Cas13b	Up to 80% C to U editing achieved using modified ADAR2DD.	(Abudayyeh et al., 2019)
LEAPER	$ADAR1_{DD}$	Engineered ADAR recruiting sequences	ADAR-recruiting RNA delivered through viral vector or plasmid or ASO to recruit endogenous ADAR for base editing.	Up to 80% editing efficiency achieved when ADAR-recruiting RNA delivered by recruiting endogenous ADAR.	(Qu et al., 2019)
REPAIRx	$ADAR2_{DD}$	CasRx	$ADAR2_{DD}$ inserted intradomain of CasRx	Up to 80% A to I editing, with intradomain ADAR2DD at position 558 of CasRx.	(Liu et al., 2020)
CIRTS	$ADAR2_{DD}$	TBP6.7	Modular RNA targeting system with $ADAR2_{DD}$ delivered with TBP6.7 and β-defensin	40% recovery of luciferase reporter was observed, when premature stop codon was targeted.	(Rauch et al., 2019)
MCP-ADAR	$ADAR2_{DD}$	MCP binding hairpin	MS2 coat protein (MCP) fused to deaminase domain of ADAR1/2, guided by antisense	Up to 50% editing efficiency achieved when targeting endogenous *RAB7A.*	(Katrekar et al., 2019)

			domain with two MS2 hairpins		
Exo-ADAR	$ADAR2_{DD}$	GluR2 ADAR binding motif	Recruiting domain of ADAR, GluR2, enhanced amd optimized to recruit endogenous/exogenous ADAR.	Up to 40% editing efficiency achieved when targeting endogenous *RAB7A.*	(Katrekar et al., 2019)
CLUSTER-gRNA	ADAR1 p110	Cluster of recruitment sequences with gRNA	Short (10-20nt) recruitment sequences incorporated adjacent to specificity domain to increase efficiency and reduce off-target effects.	Incorporating 3 recruitment sequences with gRNA led to 10% restoration of dual luciferase in wildtype mice.	(Reautschnig et al., 2022)

CIRTS: CRISPR-inspired RNA targeting system; REPAIR: RNA editing for programmable A to I replacement; RESCUE: RNA editing for specific C-to-U exchange; LEAPER: Leveraging endogenous ADAR for programmable editing of RNA.

Table 6. Summary of the current IRD mouse models with G>A or T>C transitions listed in the Mouse Genome Informatics (MGI) database adapted from Collin et al. to Prevalence of families affected in the UK sourced from Pontikos et al.

Mouse Gene	Human Gene	Human Phenoytpe	Allele Name	cDNA Change	Protein Change	Families Affected (%)
Aipl1	*AIPL1*	LCA, RP, CRD	tvrm119	c.276+5G>A	p.Val33_Ile92del	0.34
Rpgrip1	*RPGRIP1*	LCA, CRD	tvrm111	c.813+1G>A	Splice	0.23
Ush1c	*USH1C*	USH1C, RP	tm1Bkts	c.216G>A	Splice	0.34
Cngb3	*CNGB3*	ACHM	cpfl10	c.692G>A	p.Arg231His	1.53
Gnat2	*GNAT2*	ACHM	cpfl3	c.598G>A	p.Asp200Asn	0.04
Pde6a	*PDE6A*	RP	nmf282	G>A	p.Val685Met	0.38
Pde6b	*PDE6B*	RP	atrd2	G>A	Splice	0.73
Rho	*RHO*	RP	Noerg1	c.329G>A	p.Cys110Tyr	2.83
Rho	*RHO*	RP	tm1.1Eye	c.568G>A	p.Asp190Asn	2.83
Rho	*RHO*	RP	tm2.1Kpal	c.448G>A	p.Glu150Lys	2.83
Nmnat1	*NMNAT1*	LCA	imh	c.25G>A	p.Val9Met	0
Pex1	*PEX1*	Zellweger syndrome	tm1.1Sjms	c.2531G>A	p.Gly844Asp	0
Mitf	*MITF*	RP	Mi-b	c.731G>A	p.Gly244Glu	0
Mitf	*MITF*	RP	Mi-vit	c.664G>A	p.Asp222Asn	0
Alms1	*ALMS1*	Alstrom syndrome	tvrm102	c.1080+2 T>C	Splice	0.11
Tulp1	*TULP1*	RP14, LCA15	tvrm124	T>C	Splice	0.27
Grk1	*GRK1*	Oguchi disease	tvrm207	c. 1088T>C	p. Leu363Pro	0
Pde6a	*PDE6A*	RP	tvrm58	T>C	p.Leu638Pro	0.38
Pde6b	*PDE6B*	RP	rd1-2J	c.1976T>C	p.Leu659Pro	0.73
Rho	*RHO*	RP	R3	c.553T>C	p.Cys185Arg	2.83
Opa3	*OPA3*	MGCA3, Costeff syndrome	m1Votr	c.365T>C	p.Leu122Pro	0
Rpe65	*RPE65*	LCA	tvrm148	c.686T>C	p.Phe229Ser	1.22
Cacna1f	*CACNA1F*	CSNB, CRD	tm1.2Sdie	c.2267T>C	p.Ile756Thr	1.07
Reep6	*REEP6*	RP	Em2Ruch	c.404T>C	p.Leu135Pro	0.08

Ctnna1	*CTNNA1*	LCA, BSPD	tvrm5	c. 1307T>C	p. Leu436Pro	0
Rs1	*RS1*	XLRS	tmgc1	c.675+2T>C	Splice	3.18

ACHM: Achromatopsia, BSNB: Butterfly-shaped pattern dystrophy, CSNB: Congenital stationary night blindness, CRD: Cone-rod dystrophy, LCA: Leber's congenital amurosis, MGCA3: 3-methylglutaconic aciduria type 3, RP: Retinitis pigmentosa, XLRS: X-linked Retinoschisis.

Figure legends

Figure 1. Timeline of major developments in the field of RNA biology. Since the discovery of RNA in the 1890s, several landmark studies have been carried out over the 20th and early 21st century to elucidate the various functions of RNA. To date, 33 scientists have been awarded Nobel prizes for their work in the field of RNA biology. Most recently, Jennifer Doudna and Emmanuelle Charpentier collected their 2020 Nobel Prize in Chemistry for the development of the CRISPR-Cas gene editing technology.

Figure 2. The different mechanisms of action for antisense oligonucleotides (ASOs). After ASO binding to RNA target, RNase H may be recruited to cleave target RNA in RNase H mediated catalysis. Alternatively, ASO binding may merely act to provide steric hindrance against the binding of ribosomes to inhibit initiation of translation. Splicing factors may also be prevented from acting on target RNA to restore correct splicing of RNA.

Figure 3. Schematic of RNA interference mechanism of action from siRNA, shRNA and miRNA. After expression of a double-stranded RNA (dsRNA) molecule as pre-miRNA from the nucleus or pre-siRNA from the cytoplasm, the dsRNA is processed to miRNA or siRNA molecules through the Dicer enzyme. The processed RNA can then associate with a RNA-induced silencing complex (RISC) containing a Agonaute protein. Upon association with the RISC complex, the passenger strand (in blue) of the processed RNA is degraded and the complex is activated for RNA silencing. The complement strand then guides binding to target RNA, which the Agonaute protein cleaves to achieve RNA silencing.

Figure 4. Schematic of CRISPR-Cas13 mediated ssRNA cleavage. (A) Domain structure of the various Cas13 enzymes. Recognition (REC) and nuclease (NUC) domains are denoted. Cas13 enzymes function through dual-HEPN domains, that are activated upon binding to single-stranded (ssRNA) (B) CRISPR-Cas13 mechanism of action. CRISPR RNA (crRNA) sequences are acquired by the host bacteria and inserted between direct repeat (DR) sequences to form a CRISPR array. These sequences, when transcribed, form pre-crRNA which are matured upon cleavage by Cas13. Mature crRNA in association with Cas13 can then bind to target RNA sequences. This activates Cas13 activity, leading to a confirmational change that produces a 'cleavage pocket' in between the HEPN domains. Target RNA is then cleaved within the cleavage pocket. NTD, N-terminal domain. Hel, Helical. HEPN, Higher Eukaryotic and Prokaryotic Nucleotide-binding. IDL, interdomain linker.

Figure 5. CRISPR/Cas-mediated gene editing of retinal cells *in vivo*. Dual-viral suspension of AAV2-SpCas9 and AAV2-sgRNA was used (A). sgRNA plasmids also expressed mCherry, and the size of the cassettes packaged by AAV2 is displayed. (B) Representative retinal montages from Thy1-yellow fluorescent protein (YFP) mice exposed *in vivo* to our dual AAV2 plasmid system carrying SpCas9 and either control (LacZ) sgRNA or sgRNAs targeting YFP. Scale bar: 500 μm. (C) Overall, the proportion of mCherry-expressing cells (mCherry+), which lacked YFP (YFP-), was higher in SpCas9/YFP sgRNA-treated eyes. (D) The averaged ERG waveforms at selected intensities in LacZ-sgRNA–treated (n = 6, blue) and contralateral eyes (n = 6, black). The average photoreceptoral (a-wave), bipolar cell (b-wave), amacrine cell (oscillatory potentials, OPs), and ganglion cell (scotopic threshold response, STR) amplitude in LacZ sgRNA-treated relative to contralateral control eyes (%, ±SEM) is displayed. (E) Averaged ERG waveforms at selected intensities for YFP sgRNA-treated (n = 6, red) and contralateral (n = 6, black) eyes. The average a-wave, b-wave, OPs, and STR amplitude in YFP sgRNA-treated relative to contralateral control eyes (%, ±SEM) is displayed. Figure reproduced from Hung et al., 2016. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; ITR; inverted

terminal repeat; pMecp2, truncated methyl-CpG-binding protein 2 promoter; HA, hemagglutinin tag; NLS, nuclear localization signal; spA, synthetic polyadenylation signal; U6, Pol III promoter; sgRNA, single guide RNA; hSyn1, human synapsin 1 promoter; mCherry, monomeric cherry fluorescent protein; KASH, Klarsicht ANC1 Syne homology nuclear transmembrane domain; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; bGHpA, bovine growth hormone polyadenylation signal.

Figure 6. AAV-based RNA knockdown using Cas13. (A) Domain structure of single-AAV constructs for RNA silencing against neovascular ocular disease. Single AAV constructs can be designed for single (VEGF) or multiplexed (multiple VEGF or VEGF and PDGF) gene targeting. (B) Schematic of AAV-based RNA silencing with Cas13. AAV carrying CRISPR-Cas13 construct enter cells through endosomal encapsulation and upon escape, enter the nucleus to undergo uncoating and release their genetic cargo. Guide RNA and Cas13 enzymes are encoded from the delivered genetic material to form the functional CRISPR-Cas13 complex. Guide RNA is processed by Cas13 before it binds to target mRNA, activating Cas13 for targeted RNA cleavage.

Figure 7. RNA base editing of mutant mCherry. (A) Domain structure of dCas13X.1-ADAR2$_{DD}$ and CIRTS-ADAR2$_{DD}$ constructs is shown on the left, with mechanism of action against target mRNA on the right. (B) Nonsense mutation (G>A) in mutant mCherry abolishes fluorescence. Specific A>G edit reverses mutation to recover fluorescence. dCas13X.1-ADAR2$_{DD}$ outperformed CIRTS-ADAR2$_{DD}$ in base editing of mCherry in HEK293FT cells. Scale bar: 10µm.

Figure 8. Schematic of pipeline for RNA base editing therapies against IRD mutations. (A) Genetic testing of IRD patients will allow development of appropriate RNA base editors. The base editors may be validated in vitro in patient-derived iPSCs through retinal organoids. Validated RNA base editors may then be developed for patient-specific gene therapy. (B) Table of most common G>A and T>C mutations in IRDs from the Leiden Open Variation Database (LOVD) adapted from Fry et al.

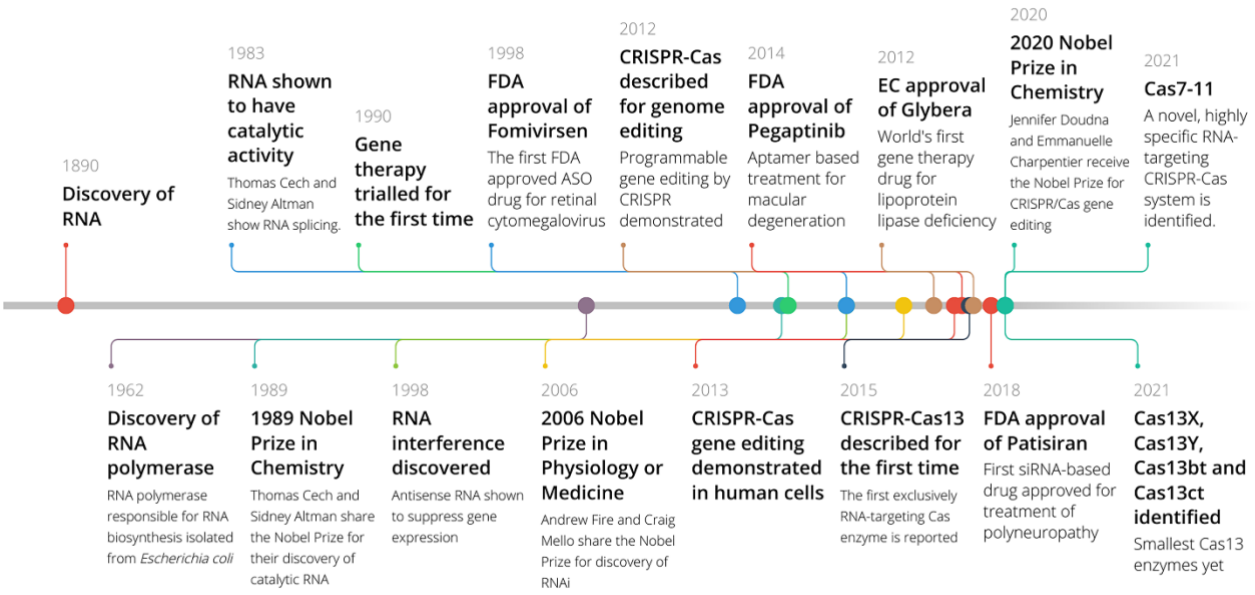


Figure 1. Timeline of major developments in the field of RNA biology. Since the discovery of RNA in the 1890s, several landmark studies have been carried out over the 20th and early 21st century to elucidate the various functions of RNA. To date, 33 scientists have been awarded Nobel prizes for their work in the field of RNA biology. Most recently, Jennifer Doudna and Emmanuelle Charpentier collected their 2020 Nobel Prize in Chemistry for the development of the CRISPR-Cas gene editing technology.

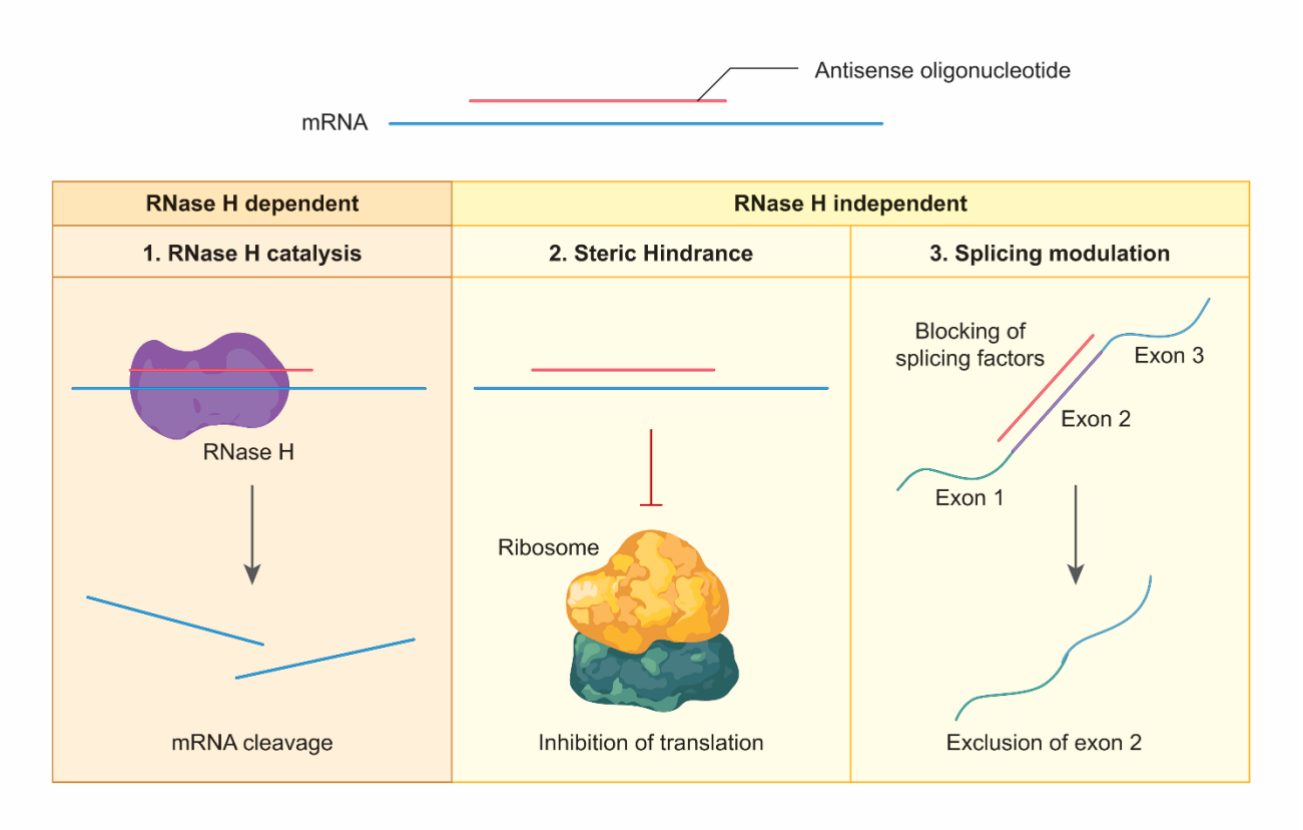


Figure 2. The different mechanisms of action for antisense oligonucleotides (ASOs). After ASO binding to RNA target, RNase H may be recruited to cleave target RNA in RNase H mediated catalysis. Alternatively, ASO binding may merely act to provide steric hindrance against the binding of ribosomes to inhibit initiation of translation. Splicing factors may also be prevented from acting on target RNA to restore correct splicing of RNA.

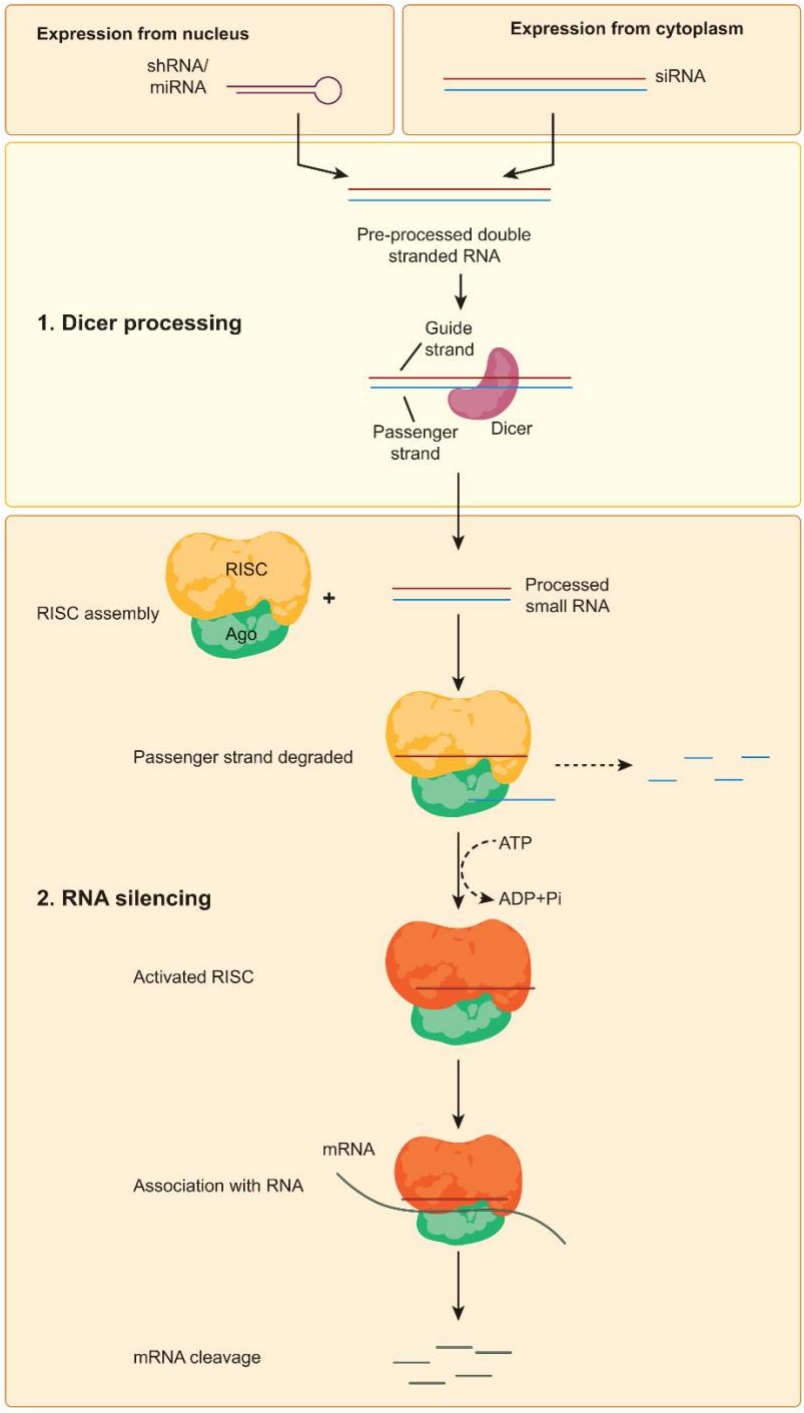


Figure 3. Schematic of RNA interference mechanism of action from siRNA, shRNA and miRNA. After expression of a double-stranded RNA (dsRNA) molecule as pre-miRNA from the nucleus or pre-siRNA from the cytoplasm, the dsRNA is processed to miRNA or siRNA molecules through the Dicer enzyme. The processed RNA can then associate with a RNA-induced silencing complex (RISC) containing a Agonaute protein. Upon association with the RISC complex, the passenger strand (in blue) of the processed RNA is degraded and the complex is activated for RNA silencing. The complement strand then guides binding to target RNA, which the Agonaute protein cleaves to achieve RNA silencing.

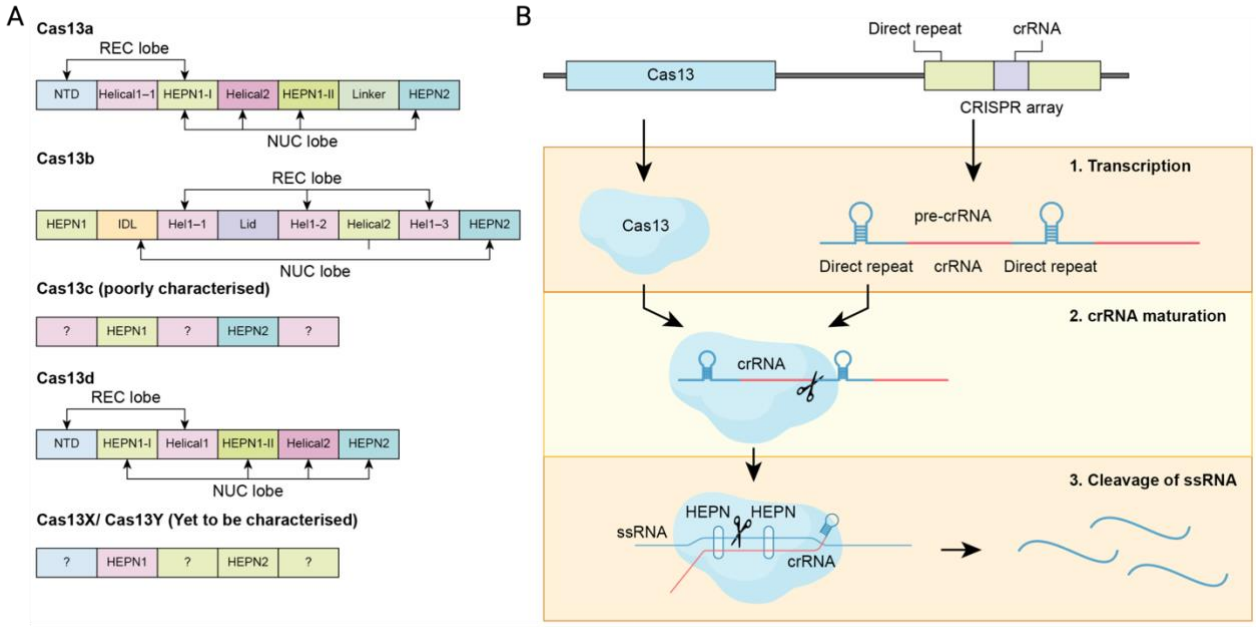


Figure 4. Schematic of CRISPR-Cas13 mediated ssRNA cleavage. (A) Domain structure of the various Cas13 enzymes. Recognition (REC) and nuclease (NUC) domains are denoted. Cas13 enzymes function through dual-HEPN domains, that are activated upon binding to single-stranded (ssRNA) (B) CRISPR-Cas13 mechanism of action. CRISPR RNA (crRNA) sequences are acquired by the host bacteria and inserted between direct repeat (DR) sequences to form a CRISPR array. These sequences, when transcribed, form pre-crRNA which are matured upon cleavage by Cas13. Mature crRNA in association with Cas13 can then bind to target RNA sequences. This activates Cas13 activity, leading to a confirmational change that produces a 'cleavage pocket' in between the HEPN domains. Target RNA is then cleaved within the cleavage pocket. NTD, N-terminal domain. Hel, Helical. HEPN, Higher Eukaryotic and Prokaryotic Nucleotide-binding. IDL, interdomain linker.

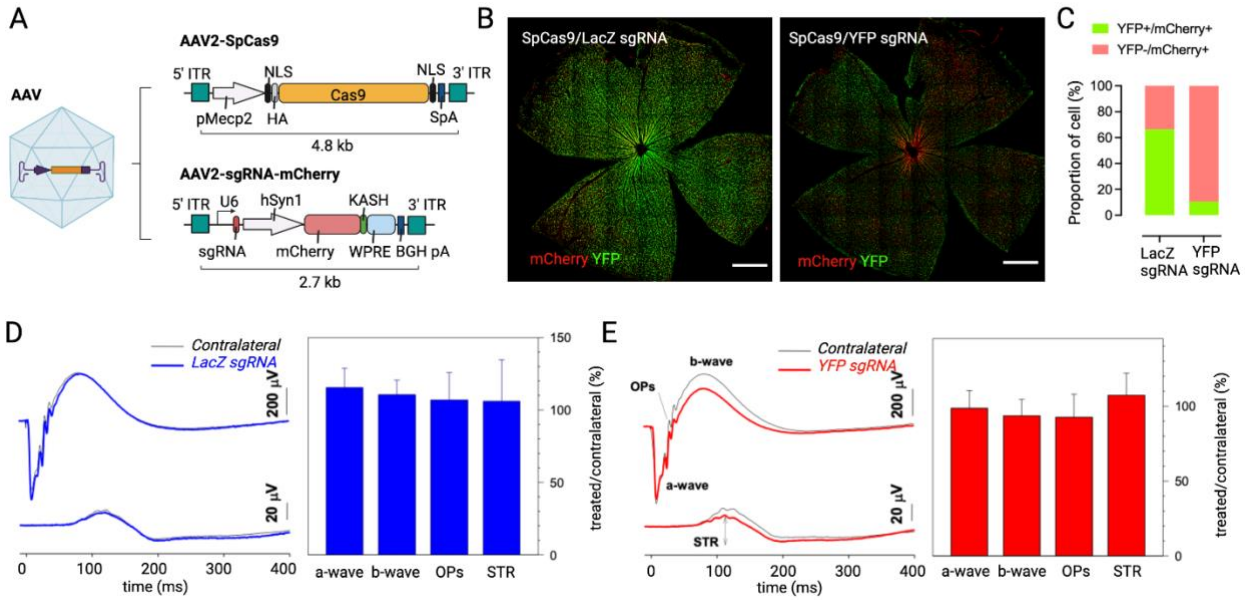


Figure 5. CRISPR/Cas-mediated genome editing of retinal cells *in vivo*. AAV2-SpCas9 and AAV2-sgRNA was used (A). sgRNA plasmids also expressed mCherry, and the size of the cassettes packaged by AAV2 is displayed. (B) Representative retinal montages from Thy1-yellow fluorescent protein (YFP) mice exposed *in vivo* to our dual AAV2 plasmid system carrying SpCas9 and either control (LacZ) sgRNA or sgRNAs targeting YFP. Scale bar: 500 µm. (C) Overall, the proportion of mCherry-expressing cells (mCherry+), which lacked YFP (YFP-), was higher in SpCas9/YFP sgRNA-treated eyes. (D and E) The averaged ERG waveforms at selected intensities in LacZ sgRNA-treated (n = 6, blue) or YFP sgRNA-treated (n = 6, red) and their contralateral eyes (n = 6, black). The average photoreceptoral (a-wave), bipolar cell (b-wave), amacrine cell (oscillatory potentials, OPs), and ganglion cell (scotopic threshold response, STR) amplitude in LacZ sgRNA-treated or YFP sgRNA-treated relative to contralateral control eyes (%, ±SEM) is displayed. Figure reproduced from Hung et al., 2016. Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; ITR; inverted terminal repeat; pMecp2, truncated methyl-CpG-binding protein 2 promoter; HA, hemagglutinin tag; NLS, nuclear localization signal; spA, synthetic polyadenylation signal; U6, Pol III promoter; sgRNA, single guide RNA; hSyn1, human synapsin 1 promoter; mCherry, monomeric cherry fluorescent protein; KASH, Klarsicht ANC1 Syne homology nuclear transmembrane domain; WPRE, woodchuck hepatitis virus posttranscriptional regulatory element; bGHpA, bovine growth hormone polyadenylation signal.

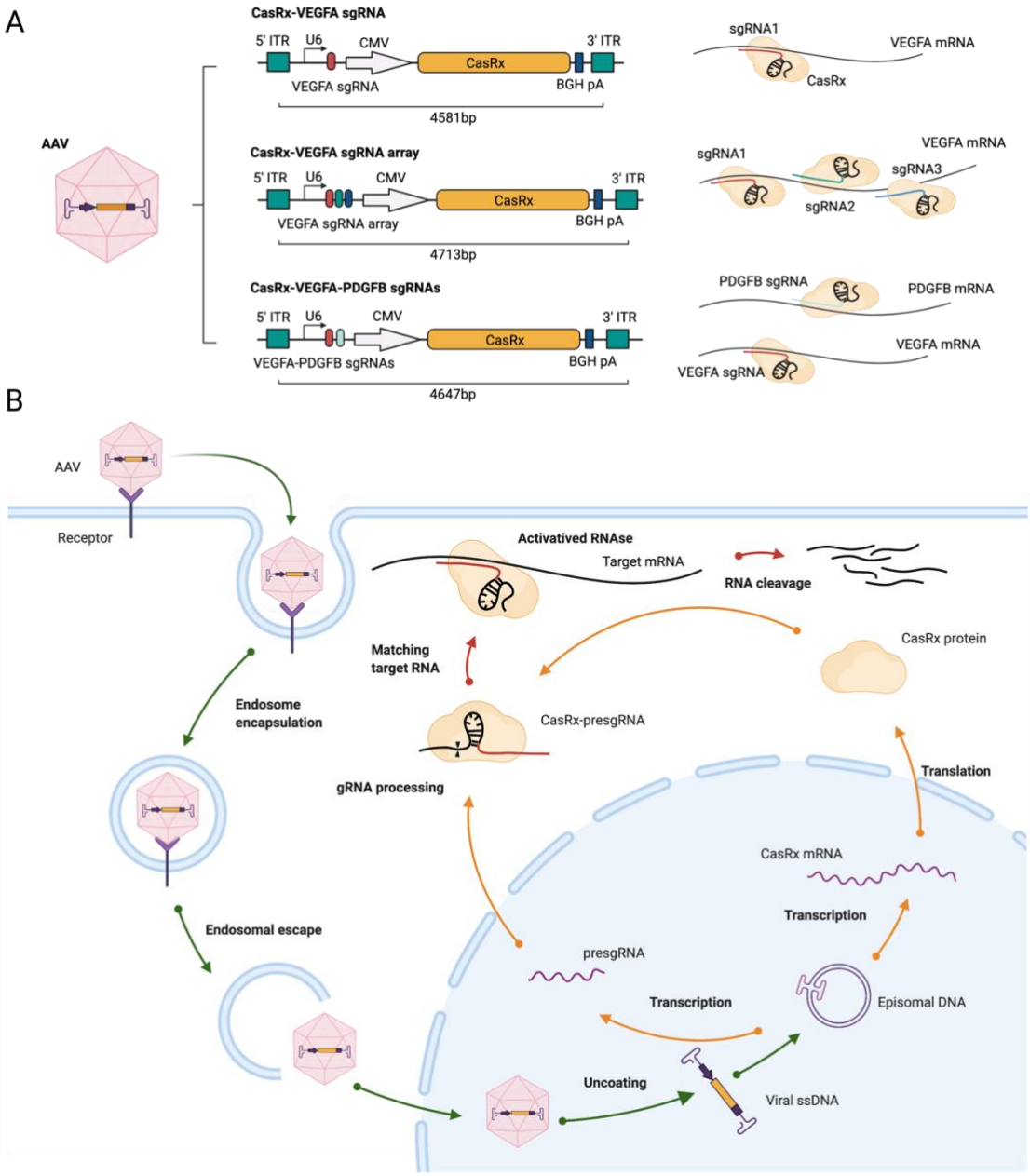
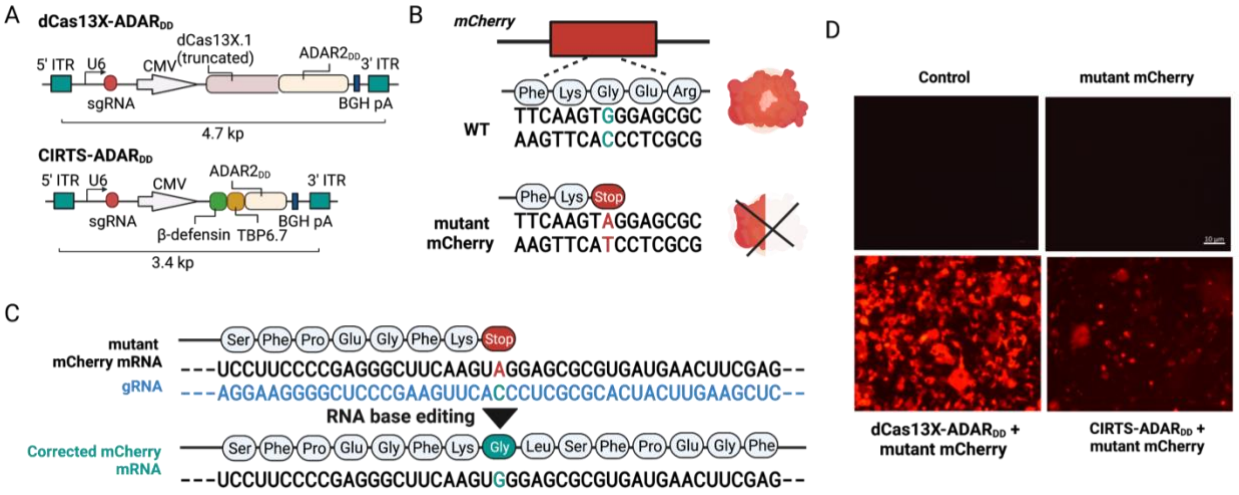


Figure 6. AAV-based RNA knockdown using Cas13. (A) Domain structure of single-AAV constructs for RNA silencing against neovascular ocular disease. Single AAV constructs can be designed for single (VEGF) or multiplexed (multiple VEGF or VEGF and PDGF) gene targeting. (B) Schematic of AAV-based RNA silencing with Cas13. AAV carrying CRISPR-Cas13 construct enter cells through endosomal encapsulation and upon escape, enter the nucleus to undergo uncoating and release their genetic cargo. Guide RNA and Cas13 enzymes are encoded from the delivered genetic material to form the functional CRISPR-Cas13 complex. Guide RNA is processed by Cas13 before it binds to target mRNA, activating Cas13 for targeted RNA cleavage.



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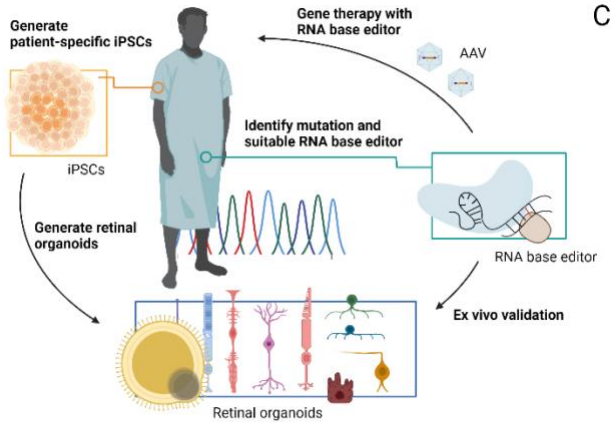
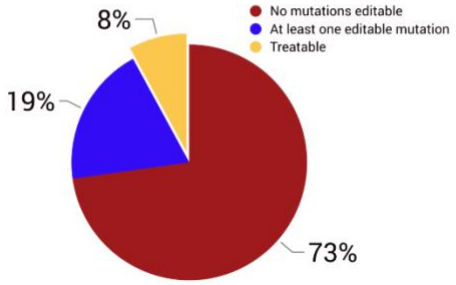
A



B



C

Gene	Phenotype	Genotype
ABCA4	Stargardt disease	c.3259G>A; c.3190G>A
		c.2625T>C;6320G>A
		c.4919G>A
		c.3259G>A
		c.5836-20G>A; c.4685T>C
USH2A	Usher syndrome 2A	c.10073G>A
		c.5776+1G>A
NMNAT1	Leber congenital amaurosis	c.769G>A
OTX2	Pattern dystrophy	c.259 G>A
PRPH2	Macular dystrophy	c.515G>A
		c.623G>A
RHO	Retinitis pigmentosa	c.553T>C
		c.488G>A
PDE6B	Retinitis Pigmentosa	c.1580T>C
TRPM1	Congenital stationary night blindness	c.296T>C; c.1871G>A
HK1	Retinitis Pigmentosa	c.2551G>A
		c.2539G>A
BBS1	Bardet-Biedl syndrome	c.1169T>C
MT-ND1	Leber's hereditary optic neuropathy	m.3460G>A
SNRNP200	Retinitis Pigmentosa	c.2359G>A
OPA1	Optic atrophy	c.1469G>A
MT-ND4	Leber's hereditary optic neuropathy	m.11778G>A
ADGRV1	Usher syndrome 2C	c.5901T>C; c.13232-1G>A

Figure 8. Schematic of pipeline for RNA base editing therapies against IRD mutations. (A) Genetic testing of IRD patients will allow development of appropriate RNA base editors. The base editors may be validated in vitro in patient-derived iPSCs through retinal organoids. Validated RNA base editors may then be developed for patient-specific gene therapy. (B) Proportion of patients who carry treatable mutations from an Australian cohort (n = 441), with phenotype known and iPSCs available. (C) List of editable mutations from an Australian cohort (n = 441), along with specific gene and patient phenotype.