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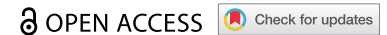


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REVIEW



## Is subretinal AAV gene replacement still the only viable treatment option for choroideremia?

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### ABSTRACT

**Introduction:** Choroideremia is an X-linked inherited retinal degeneration resulting from mutations in the *CHM* gene, encoding Rab escort protein-1 (REP1), a protein regulating intracellular vesicular transport. Loss-of-function mutations in *CHM* lead to progressive loss of retinal pigment epithelium (RPE) with photoreceptor and choriocapillaris degeneration, leading to progressive visual field constriction and loss of visual acuity. Three hundred and fifty-four unique mutations have been reported in *CHM*. While gene augmentation remains an ideal therapeutic option for choroideremia, other potential future clinical strategies may exist.

**Areas covered:** The authors examine the pathophysiology and genetic basis of choroideremia. They summarize the status of ongoing gene therapy trials and discuss *CHM* mutations amenable to other therapeutic approaches including CRISPR/Cas-based DNA and RNA editing, nonsense suppression of premature termination codons, and antisense oligonucleosides for splice modification. The authors undertook a literature search in PubMed and NIH Clinical Trials in October 2020.

**Expert opinion:** The authors conclude that AAV-mediated gene augmentation remains the most effective approach for choroideremia. Given the heterogeneity of *CHM* mutations and potential risks and benefits, genome-editing approaches currently do not offer significant advantages. Nonsense suppression strategies and antisense oligonucleotides are exciting novel therapeutic options; however, their clinical viability remains to be determined.

### ARTICLE HISTORY

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### KEYWORDS

Choroideremia; CRISPR; AAV; gene therapy; antisense oligonucleotides; nonsense suppression; REP1; CHM

## 1. Introduction

### 1.1. Choroideremia: pathophysiology and clinical features

Choroideremia is an inherited retinal degeneration that affects approximately 1 in 50,000 people worldwide. It is caused by mutations in the *CHM* gene, located on Xq21.2 (OMIM \*300390). *CHM* encodes Rab-escort protein 1 (REP1), a polypeptide involved in the prenylation pathway of Rabs, GTPase-associated proteins essential for normal intracellular vesicular transport. Binding of REP1 to its Rab facilitates the addition of a geranylgeranyl group to the Rab by Rab geranylgeranyl-transferase (RabGGTase) in a prenylation reaction. REP1 is subsequently involved in delivery of the prenylated Rab to a specific membrane [1]. Cellular models suggest that pathogenic mutations in REP1 result in aberrant binding to and thus under-prenylation of Rabs [2,3], an observation supported by animal models of choroideremia showing that Rab under-prenylation is associated with disordered phagocytic function of the retinal pigment epithelium (RPE), and subsequent RPE degeneration [1,4]. Whether retinal degeneration in choroideremia is primarily driven by RPE or photoreceptor loss is a source of some debate. Conditional knockout of REP1 in

the RPE and photoreceptors in mice suggest that photoreceptor dysfunction and loss may occur independently of RPE dysfunction [5], but RPE degeneration nonetheless accelerates photoreceptor loss [6]. OCT studies in choroideremia patients indicate that photoreceptor loss generally follows RPE loss to give rise to stereotypical 'islands' of surviving retina, and choriocapillaris atrophy generally occurs later on in the disease process [7]. Accordingly, structural changes typical of photoreceptor stress secondary to RPE disease have been observed in the form of outer retinal tubulations [7,8]. Adaptive optics imaging in choroideremia indicates that a degree of photoreceptor structural abnormality is present within the islands of surviving RPE [9] and cone photoreceptors may be able to survive a short distance beyond the edge of RPE degeneration [10]. However, it remains unclear to what extent these stressed or structurally abnormal photoreceptors are amenable to therapeutic rescue.

Although REP1 is ubiquitously expressed throughout the body, its deficiency through genetic mutations in *CHM* only manifests as disease in the eye. The lack of systemic effects is thought to be due to redundancy of Rab prenylation activity provided by REP2, a homologous protein encoded by the *CHM*-like (*CHML*) X-linked retrogene located on chromosome

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**Article highlights**

- Choroideremia is a loss of function disease. The majority of mutations in choroideremia are truncating, nonsense mutations leading to loss of protein product.
- Gene replacement therapy is the most logically appealing form of therapy for choroideremia. AAV2-REP1 is currently in phase 3 trials and remains the most promising treatment likely to be available in the near future.
- CRISPR-mediated base and prime editing are exciting new therapeutic options with widespread applicability, programmability and flexibility. Over 50% of mutations in choroideremia are single-base substitutions: of the remainder, only 14% (comprising large deletions, duplications or insertions) are not amenable to prime editing. Nonetheless, due to the heterogeneity of mutations in *CHM*, currently, they do not represent a cost-effective alternative to gene replacement.
- RNA editing, antisense oligonucleotides and nonsense suppression strategies are therapies that do not rely on permanent editing of the genome. However, so far, there is a lack of long-term data for these therapies, and there is also a lack of potential therapeutic sites for antisense oligonucleotides.

This box summarizes key points contained in the article.

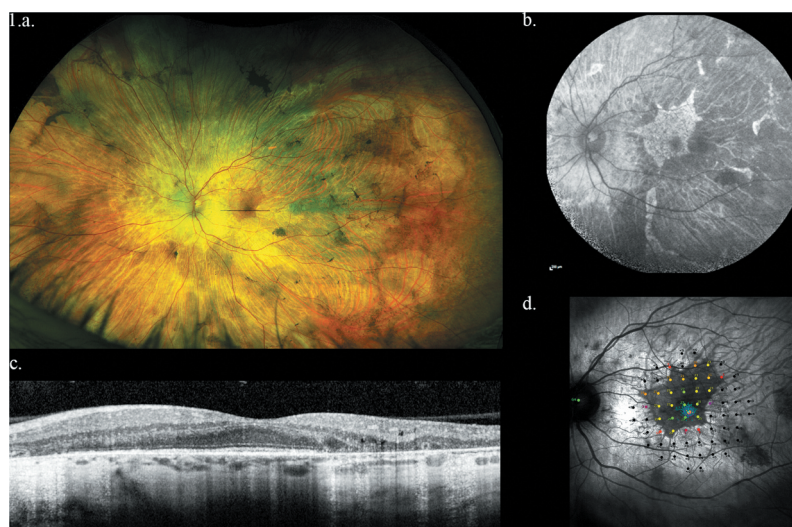
1q42 [11]. It remains unclear why this compensation mechanism is insufficient in the outer retina, leading to slow degeneration of the RPE and photoreceptors [12]. It is established that certain Rabs (e.g. Rab27a) compete for REP1 in preference to REP2, and that a Rab 'prenylation hierarchy' exists [13] where certain Rabs bind more efficiently to both REP1 and REP2, thus out-competing lower ranking Rabs which become underprenylated in the absence of REP1 [2,13,14]. This, combined with the high phagocytic demands placed on RPE by continuous photoreceptor outer segment turnover, may lead to errors in vesicular trafficking and cumulative cellular toxicity [15].

Choroideremia typically presents in males with nyctalopia during childhood, followed by progressive and disabling loss of peripheral vision beginning in the late teens. The area of surviving RPE generally follows a steady exponential decay with a half-life of around 5.5 years (95% CI: 5.0–6.1) [12]. In end-stage disease, usually by the fifth to seventh decade of life, central vision becomes extinguished as the centripetal RPE degeneration encroaches on the fovea (Figure 1). Female carriers of *CHM* mutations are usually asymptomatic although wide variation may be seen in retinal status and visual function [8,16]. This is likely the consequence of random inactivation of X chromosomes in females giving rise to mosaics of healthy and diseased RPE cells in the retina [17]. As the majority of choroideremia patients harbor null mutations in *CHM*, there is limited evidence for phenotype-genotype correlation [18]. However, rare cases of splicing defects have been reported in which residual *CHM* mRNA transcripts have been associated with unusually slow rates of disease progression [19].

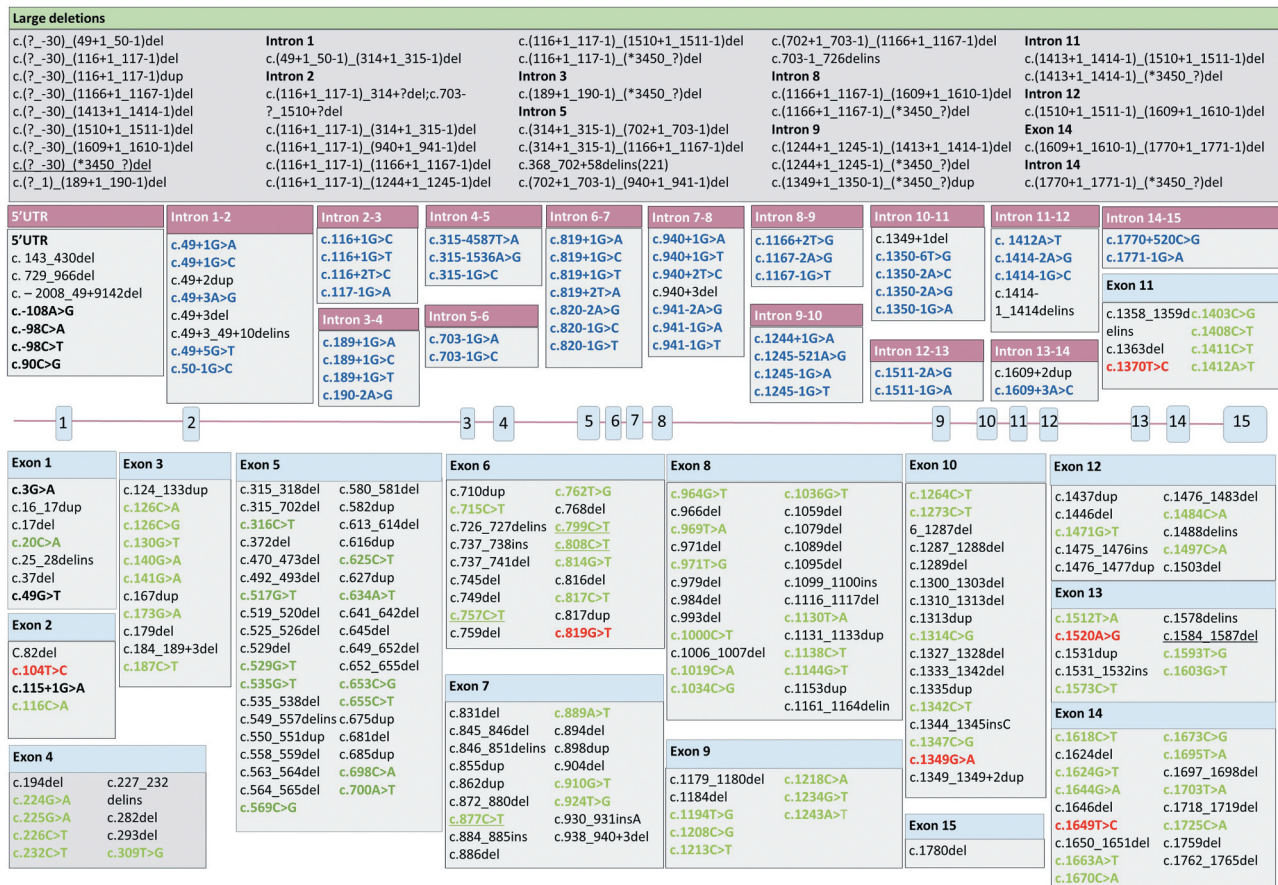
Choroideremia has several features that make it an attractive target for genetic therapies. The disease is monogenic with an easily recognizable phenotype and X-linked family history. The disease progression is relatively slow, thus providing a long therapeutic window for intervention. As a disease that affects the young, the potential benefits of treatment over a lifetime are substantial. As a disease that affects only the eye, a relatively immune privileged site, targeted local therapy would be feasible.

## 1.2. Potential therapeutic targets

The *CHM* gene contains 15 exons (OMIM) and is 5,442 bp in length, with the last exon making up the majority of the transcript length, of which 3,450 bp encodes the 3'-untranslated region (UTR) (Figure 2(a)). It encodes REP1, a 653 amino acid (aa) protein. The Leiden Open Variation Database lists 628 *CHM* public variants, of which 545 are classed as pathological



**Figure 1.** Representative left eye images of 32 year old male with *CHM* mutation. (a) Widefield optos color image showing baring of sclera and peripheral pigmentation. (b) Macular fundus autofluorescence showing the central area of relative RPE preservation. (c) OCT image showing loss of outer retina with central island of preservation. (d) Microperimetry data showing central preservation of retinal sensitivity with sharply demarcated sensitivity drop-off corresponding to island of surviving RPE.



**Figure 2a.** (a) Structure of the *CHM* gene annotated with pathogenic variants. Intronic mutations have been color coded with a pink header, exonic mutations with a blue header. Single-base substitutions are shown in bold: of these, single-base substitutions resulting in missense mutations are colored green; and splicing defects are colored purple. The five most common described variants are underlined (c.(?-30)\_(\*3450?)del ( $n = 20$ ), c.757 C > T ( $n = 18$ ), c.799 C > T ( $n = 16$ ), c.1584\_1587del ( $n = 11$ ), 808 C > T and 877 C > T ( $n = 10$  each)).

or likely pathological, and 354 are unique variants. Mutations have been reported within the promoter region [20,21], introns [22,23], and exons [18]. The vast majority of the exonic variants are nonsense mutations which result in an absence of or truncation of the protein. Missense mutations associated with a clinical phenotype remain rare, but at least three have been identified. These have been associated with a reduction in REP1 expression, presumably due to destabilization of protein structure, or a functional reduction in prenylation activity [24–26]. One theory advanced to explain this is that missense mutations in REP1 may be well tolerated, based on the fact that REP2 shares only around 75% amino acid identity with REP1 yet retains similar affinity for Rab binding [1,14]. It has been also been postulated that as a chaperone protein, REP1 folding may be influenced by its interaction with the Rab ligand rather than post-translational modification, thus rendering it more tolerant of single amino acid changes [18].

Pathogenic *CHM* variants occur primarily in exonic DNA (66%), of which just over half are due to substitutions. Of these substitutions, the vast majority (over 90%) result in a premature termination codon (PTC). The next most common region for pathogenic mutations involve splicing/intronic regions (18%), followed by deletions (15%) which commonly result in no protein product being formed [27] (Figure 2(b)).

The current arsenal of potential therapeutic options for choroideremia include gene augmentation, CRISPR/Cas-mediated base-editing, prime-editing, nonsense-suppression and antisense oligonucleotides. This review discusses the potential applications of each approach to choroideremia-associated targets, their impact, feasibility, and key advantages and disadvantages. The authors undertook a literature search in PubMed and NIH Clinical Trials between October 2020 and January 2021. Search terms used included choroideremia, *CHM*, REP1, AAV, AON, nonsense-suppression, CRISPR.

## 2. Gene augmentation therapy

Gene augmentation for choroideremia is an appealing therapeutic strategy. It is applicable to any *CHM* mutation, independent of its location. It is also applicable to large or multiple deletions, which would be less amenable to site-specific strategies such as base- or prime-editing. It is important to note that there has been no evidence for any toxicity or dominant negative effect for any of these abnormal protein products in female carriers: it is also clear from carriers that partial expression of wild-type REP1 (as may be achieved through gene augmentation) is generally sufficient to preserve visual function, although retinal morphology may be affected [16,28].



**Table 1.** Summary of trials involving REP1 replacement and their status.

Clinical Trial registration	Location/Start date	Vector design	Primary end points reported
NCT01461213	University of Oxford, UK, October 2011, Phase 1/2	rAAV2-REP1 0.6–1.0 × 10 <sup>10</sup> viral particles	<i>Lancet</i> 2014 [32] <i>NEJM</i> 2016 [34] <i>Nat Med</i> 2018 [35] No reports [36]
NCT02341807	Philadelphia, USA Spark Therapeutics January 2015, Phase 1/2	AAV2-hCHM at high and low dosage	No reports [36]
NCT02077361	University of Alberta, Canada, April 2015, Phase 1/2	rAAV2-REP1 (used in University of Oxford trial NCT01461213)	<i>Am J Ophthalmol</i> [37]
NCT02553135	University of Miami, USA, September 2015 Phase 2	rAAV2-REP1 1.0 × 10 <sup>11</sup> viral particles	<i>IOVS</i> 2017 [38] <i>Am J Ophthalmol</i> 2019 [39]
NCT02671539	University of Tübingen, Germany THOR trial, January 2016, Phase 2	rAAV2-REP1 1.0 × 10 <sup>11</sup> viral particles	<i>JAMA</i> , 2019 [40] <i>Retina</i> 2020 [41]
NCT02407678	University of Oxford and Moorfields Eye Hospital, UK REGENERATE August 2016, Phase 2	Unilateral rAAV2-REP1	<i>IOVS</i> 2017 [42]
NCT03507686	Nightstar Therapeutics International GEMINI, November 2017, Phase 2	Bilateral rAAV2-REP1 1.0 × 10 <sup>11</sup> viral particles	No reports [43]
NCT03496012	Nightstar Therapeutics International, STAR December 2017, Phase 3	Unilateral rAAV2-REP1 1.0 × 10 <sup>11</sup> viral particles	No reports [44]
NCT03584165	Nightstar Therapeutics International SOLSTICE, June 2018	Observational, long-term safety follow-up of AAV2-REP1	No reports [45]
NCT04483440	4D Molecular Therapeutics, Roche Pharma and 4D-MT, June 2020, Phase 1	Intravitreal AAV (4D-R100)-hCHM dose escalation trial	No reports [31]

date has shown efficient targeting of the RPE. In a preclinical study of intravitreal AAV evolved in nonhuman primates, data on two evolved vectors NHP#9 and NHP#26 were presented. The former was injected at  $1.5 \times 10^{12}$  viral genomes which is a log unit higher than the maximal AAV2 doses used in human clinical trials and failed to transduce RPE cells, despite good inner retinal expression. Similarly, no RPE cell transduction was observed with NHP#26 even when using immunohistochemistry to detect faint GFP expression [46]. Since successful RPE transduction is almost certainly essential for treating choroideremia with gene replacement, intravitreal AAV is unlikely to deliver successful outcomes. Furthermore, intravitreal administration of AAV carries the potential development of immune and inflammatory responses which are detectable both locally and systemically, and could adversely affect visual outcomes [47,48]. Indeed, the NHP subjects in Byrne et al.'s paper were systemically immunosuppressed daily with subcutaneous ciclosporin [46].

### 3. Genomic editing

#### 3.1. Principles of CRISPR-Cas genome editing

The discovery of RNA-guided Cas9 endonucleases from prokaryotic Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) systems and their adaptation into powerful gene editing tools have revolutionized the field of molecular biology [49]. CRISPR-Cas9 systems' highly programmable and precise editing abilities have opened up a wide array of potential therapeutic genetic targets in human inherited diseases. Although the

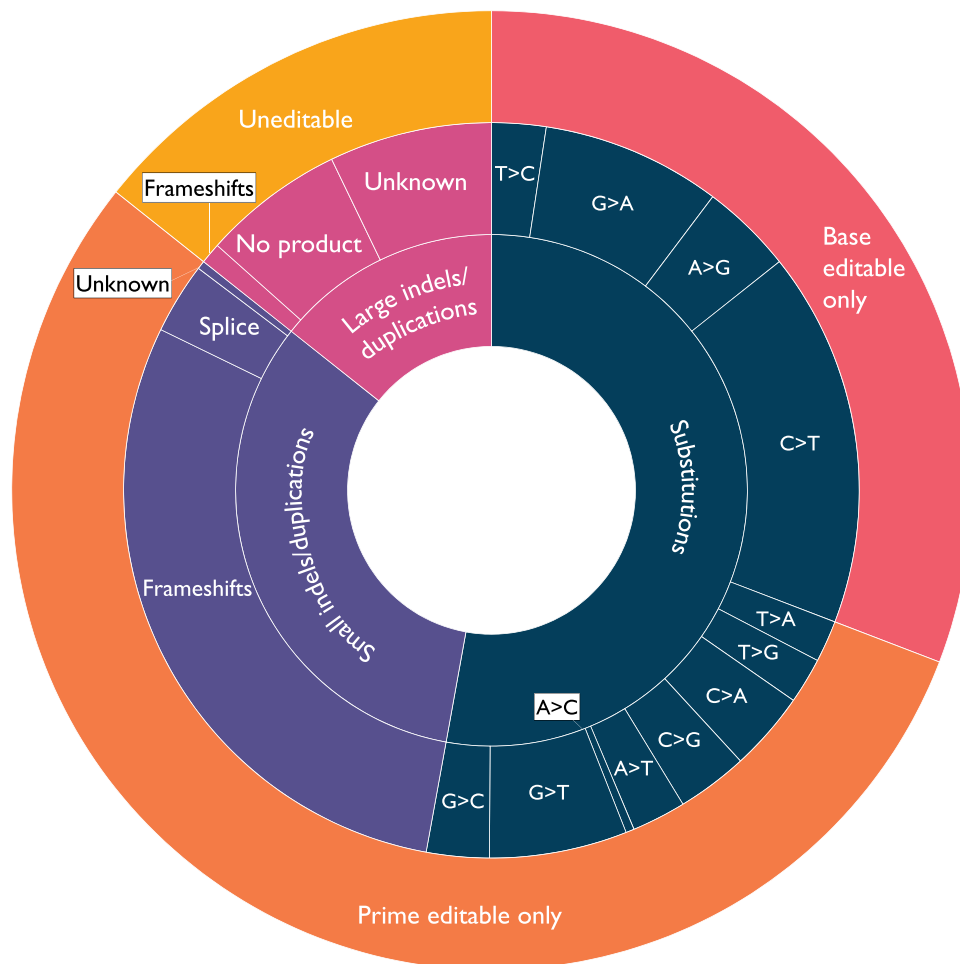
characterization of CRISPR biology and subsequent adaptation for gene editing has occurred only in this decade, the potential of CRISPR-based strategies for the treatment of inherited retinal diseases has already been rapidly and extensively realized. CRISPR/Cas9-based genome editing in mouse models has already been achieved in proof-of-principle studies as well as targeting of common mutations causing inherited retinal diseases [50–54]. Successful pre-clinical genome editing of a deep-intronic mutation in *CEP290*, which causes LCA10, has led to the first FDA-approved CRISPR genome engineering clinical trial for an inherited retinal disease, and the first delivery into a patient was achieved in early 2020 [52].

The CRISPR-Cas family originates from the bacterial innate immune system's need to recognize and destroy intruder viral DNA and can be found in a wide variety of prokaryotic species. Family members differ in mechanisms of action, but all CRISPR-Cas systems require a target-specific CRISPR RNA (crRNA) to guide it to the target sequence, and (for type II systems) a trans-activating RNA (tracrRNA), which forms a scaffold structure to interact with the Cas protein. For genome

**Table 2.** Most common substitutions in *CHM*. The top five are all C to T transitions, which lead to the creation of a STOP codon.

Mutation	Translation	Patients (n)	% of Pathogenic variants
c.757 C > T	p.(Arg253*)	18	3
c.799 C > T	p.(Arg267*)	16	3
c.808 C > T	p.(Arg270*)	10	2
c.877 C > T	p.(Arg293*)	10	2
c.715 C > T	p.(Arg239*)	7	1
	Total		11

Source: Leiden Open Variation Database (LOVD) [27].



**Figure 3.** Potential genome editing targets in *CHM*. The inner ring shows type of mutation. The middle ring shows the single-base substitutions (which overwhelmingly produce terminations, as previously described) and other consequences of the mutations (Frameshifts, splice defects, no protein product formed, or unknown). The outer ring shows the number of potential base editing targets in *CHM* (red: currently restricted to transitions, which comprise approximately 30.8% of choroideremia-associated mutations; the remaining 69.2% are not amenable to base-editing methods): the number of potential prime editing (orange: prime editing opens up the potential number of targets up to 85.7% of *CHM* variants): and remaining uneditable mutations (yellow: large insertions, deletions and duplications) Source: Leiden Open Variation Database (LOVD) [27].

editing applications, researchers have linked the naturally occurring crRNA and tracrRNA described above to form a single guide RNA (sgRNA). The Cas9:sgRNA complex scans DNA within the nucleus, searching first for the appropriate protospacer adjacent motif (PAM), a nucleotide sequence specific to each Cas family, which allows binding of the Cas protein. Subsequently, the sgRNA binds to a target sequence adjacent to the PAM, and the Cas endonuclease cleaves the nucleic acid to generate a double-strand break (DSB). In eukaryotes, Cas9-induced DSBs are repaired by DNA repair mechanisms [55]. Commonly this occurs via error-prone non-homologous end joining (NHEJ), resulting in random insertions or deletions (indels) and disruption of the target gene [56]. Alternatively, a repair template with homology to the target region can be delivered to stimulate error-free homology-directed repair (HDR), although at a lower efficiency than NHEJ-mediated repair. Nevertheless, the correction of a point-mutation by HDR has been shown to be highly inefficient, particularly in non-dividing cells, such as those present in the neuroretina. Furthermore, when Cas9 nuclease generates a DSB, this simultaneously generates undesired indels at a substantial frequency, thus introducing further unwanted mutations [57].

### 3.2. CRISPR-based DNA base editing in choroideremia

To address the limitations of HDR-mediated gene editing, CRISPR-Cas-mediated single-base pair editing systems (or 'base editing' systems) have been devised which allow for targeted restoration of single-base mutations. Two classes of DNA base editors have been described to date: cytosine base editors and adenine base editors [58,59]. DNA base editors encompass two key components. The first is an inactivated Cas enzyme (or Cas nickase, nCas9) which retains its programmable DNA binding ability, but which has lost its ability to generate DSBs. The second is a single-stranded DNA-modifying enzyme (cytidine or adenine deaminase) fused to nCas9 for targeted nucleotide alteration. Collectively, all four transition mutations (A > G, C > T, G > A and T > C) can be installed with the available CRISPR/Cas base editor systems. Recently, Kurt et al. described the engineering of two novel base editor architectures that can efficiently induce targeted C-to-G base transversions [60]. In addition, recent studies report dual-base editor systems for combinatorial editing in human cells. Together, these new base editors expand the range of DNA base editors to transversion mutations and may

allow for targeting of more complex compound edits than are currently achievable by a single DNA base editor.

Analysis of publicly available variants in the Leiden Open Variation Database (LOVD) [27] reveals that 52.8% of pathogenic variants in the *CHM* database are substitutions, with the remaining variants not amenable to base editing as they involve indels (Figure 2(b)). Despite this, 30.8% of pathogenic variants were amenable to base editing with the commonly used editors capable of editing transition variants (A > G, C > T, G > A, A > G). Of these, C > T and G > A mutations were more common, with C > T substitutions resulting in the creation of a premature stop codon the most common (Figure 3). Most of these mutations are unique within families, however, and there is no single mutation that is obviously common and easily targetable. The five most common pathogenic variants are presented in Table 2, and collectively account for around 11% of all pathogenic variants. These all arise from C > T transitions, creating a TGA (STOP) from a CGA (Arg) codon in exon 6 or 7. These mutations are amenable to editing with cytidine base editors or the recently developed RESCUE RNA editor (see below, section 3.4). Though the cost of individualized treatment may be high, it is worth considering that if a CRISPR base or prime editor can be delivered separately from its guide RNA, a single C > T base-editing construct can be used with separate, personalized guides, allowing greater therapeutic flexibility.

### 3.3. CRISPR-based prime-editing in choroideremia

Recently, a method to overcome the inability of base-editors to generate precise base-edits beyond the four transition mutations has been described by Anzalone et al. [61]. Known as prime editing, this method enables the introduction of indels and all 12 base-to-base conversions (both transitions and transversions), as well as insertions up to 44 bp and deletions up to 80 bp. As with CRISPR-mediated base editing, prime editing does not involve the creation of DSBs. Prime editors use an engineered reverse transcriptase fused to nCas9 and a prime editing guide RNA (pegRNA). Here, the pegRNA differs significantly from regular sgRNAs and plays a major role in the system's function. The pegRNA contains not only the sequence complementary to the target sequence that directs nCas9 to its target site but also an additional sequence spelling the desired sequence change, which is reverse transcribed into the genome. The first generation of prime editors (PE1) was comprised of Moloney murine leukemia virus reverse transcriptase (M-MLV RT) linked to the C-terminus of nCas9 and pegRNA, which was expressed on a second plasmid. The latest generation prime editor, using enhanced M-MLV RT, performed all possible transition and transversion mutations with average editing efficiencies of 33% ( $\pm 7.9\%$ ) [61].

As well as vastly expanding the number of potentially correctable pathogenic mutations in human DNA, prime editing has other advantages over previous CRISPR-mediated base editing approaches. Prime editors have less stringent PAM requirements due to the varied length of the reverse transcriptase template, and no 'bystander' editing. Prime editing could potentially address all pathogenic *CHM* mutations apart

from the 14% comprising large deletions, insertions or duplications (Figure 3).

Prime editing offers similar benefits as base editing but provides greater target flexibility. Though its efficiency is low, examination of REP1 mRNA transcript levels in choroideremia patients with rare slow-progressing phenotypes show they have overall mRNA levels of <1% of those of non-affected individuals, while patients with normal disease phenotypes had undetectable mRNA levels. This implies that even an editing efficiency of 1% of mRNA transcripts may give sufficient protein product to slow down disease progression [19]. There is a paucity of long-term data, but prime editing has enormous potential in addressing the range of human heritable diseases [62]. However, current base and prime editor sizes both total >6 kb, well beyond the packaging capacity of AAV. Finally, it is important to note that prime editing tools are still in their infancy, and additional *in vivo* characterization is needed before their broader use for targeting inherited retinal diseases. Indeed, the introduction of exogenous reverse transcriptase should be treated with caution due to potential enabling effects on dormant pro-viral or retro-elements.

### 3.4. CRISPR-RNA editing

Finally, CRISPR-directed RNA editing represents another novel approach to targeted correction of single nucleotide variants, in RNA rather than DNA [54]. A wide variety of approaches have been developed to edit RNA *in vitro*. Each approach currently uses a variant of the Adenosine Deaminase Acting on RNA (ADAR), naturally expressed enzymes in human cells that undertake physiological RNA editing functions. These deaminases convert adenosine bases to inosine in RNA, which is read as a guanosine in cellular processes such as translation and splicing [63]. This effectively creates an A > G edit in RNA and can be harnessed for the correction of G > A mutations. ADAR variants have also been engineered to create C > U edits: together, they can theoretically address up to 10% of known *CHM* mutations [64,65]. Harnessed for site-directed RNA editing, ADAR can be recruited to editing sites of interest by systems that link ADAR to an effector molecule and direct the ADAR-effector system with a gRNA to the base to be edited [54]. Many effectors have been developed including those based on CRISPR-Cas13 [65,66] or Cas9 systems [67], bacteriophage-derived  $\lambda$ N peptide [71] and BoxB system, aptamer-like systems such as the MS2 bacteriophage coat protein (MCP) or GluR2 system [69]. Additionally, systems that deliver only a gRNA and use only endogenously expressed ADAR have been developed [69–71], in contrast [68] to other systems that require ADAR overexpression.

RNA editing provides a number of theoretical advantages for safety in clinical translation. RNA editing is not permanent, as edited mRNAs eventually decay. A self-limiting treatment has an inherent appeal in a disease that affects the young, particularly as there is a paucity of long-term *in vivo* data for all DNA and RNA editing applications, simply by virtue of their novelty. The disadvantage of a self-limiting treatment, of course, is that repeated treatments would be required to maintain the therapeutic effect. Alternatively, a method of



continual ADAR and gRNA expression must be used to achieve ongoing on-target repair. A mismatch between ADAR and gRNA expression in such a system may result in an increase in off-target effects. On the other hand, with a self-limiting system, off-target effects are also not permanent. This may allow for reversible or titratable editing, or systems with an in-built 'off' safety switch.

While RNA editing is an exciting prospective tool to add to the gene therapy researcher's toolbox, the technology is still in its infancy. Though truncated ADAR-Cas systems have been engineered to maintain editing efficacy while meeting AAV-packaging constraints, AAV-delivery of an ADAR-Cas system has yet to be achieved. Work on targeting human mutations transfected into HEK293 cells achieved at best 35% editing efficacy: typical efficacies were only 'up to' 28% [66]. Alternate systems such as the GluR2 and MS2 systems are readily deliverable within AAV packaging constraints. Delivery of these systems via AAV *in vivo* to two mouse models have resulted in editing efficiencies of less than 5% [69]. Further work is required to understand the impact of off-target RNA editing in the transcriptome. A functional RNA editor ready for in-human trials is not a near-future possibility.

#### 4. Antisense oligonucleotides

In eukaryotic mRNA processing, splicing of pre-mRNA is directed by interactions between the spliceosome (a complex of proteins and small nuclear RNAs which facilitates intronic excision) and the 5' donor, branch, and 3' acceptor sites within each intron. Typically, the splice donor site (5' end of the intron) includes a GU sequence, followed by a relatively unconserved section which is followed by the branchpoint and a polypyrimidine tract, a sequence rich in pyrimidines, and terminating in the acceptor site at the 3'-end, usually an AG sequence. Mutations within these key areas of the intron can lead to the formation of a cryptic splice site, able to redirect the pre-mRNA's interactions with the spliceosome and resulting in the insertion of an aberrant exon.

Splice-modifying antisense oligonucleotides (AON) are short nucleotide sequences designed to restore normal splicing by redirecting pre-mRNA/spliceosome interaction. AONs are designed either to bind to the cryptic splice site and prevent it from interacting with the spliceosome, or to bind to the aberrant exon and thus encourage skipping of said aberrant exon during splicing [72]. An AON targeting the common deep-intronic mutation in *CEP290* (c.2991 + 1655A>G, p.Cys998\*) in LCA10 has been shown to be able to correct aberrant splicing in pre-clinical studies and is undergoing phase 1 clinical trial with a good safety profile and promising early efficacy data [73–75]. The trial by Cideciyan et al. is particularly encouraging because the AON was delivered by intravitreal injections with effects appearing to last several months. Moreover, it affected functional and anatomical changes in photoreceptors of some treated eyes, with improved retinal structure by optical coherence tomography (OCT), improvements in full field stimulus testing, and improvement in visual acuity in one treated patient [75].

Of the 500 or so pathogenic variants found in choroideremia, 100 are predicted to cause splicing abnormalities.

However, the vast majority of these are single nucleotide substitutions in the 5' splice donor or 3' splice acceptor sites, and therefore not amenable to the AON mechanism of splice rescue. Few potential targets have been described [76]. One attempt has been made to target the c.315–4587 T > A mutation, which creates a 98-bp pseudoexon insertion and loss of protein function. An AON was designed to target the pseudoexon and delivered *in vitro* to lymphoblast cells from two choroideremia patients. Disappointingly, despite showing almost full redirection of splicing to produce detectable levels of corrected transcript in both patient-derived lymphoblast cells, resultant REP1 expression was low. The authors have speculated that this may be due to unpredicted effects on splicing from AON binding, or possible initiation of transcript degradation caused by AON binding [77].

#### 5. Nonsense suppression strategies

Around 32% of all *CHM* mutations (and 49% of all *CHM* mutations within coding sequences) result in the formation of a premature termination codon without inducing frameshift or splicing defects. A strategy which can be applied to all PTC-generating mutations therefore has great appeal and applicability.

The generation of a PTC can result in either nonsense-mediated decay of the resultant mRNA transcript, or, if it escapes decay, the production of a truncated and nonfunctional protein product. In nonsense-mediated decay, activity is triggered by the relative location of the PTC within the mRNA sequence to the binding location of a protein complex, the exon-junction complex (EJC). The EJC normally binds approximately 20–24 nucleotides upstream of an mRNA splice-site. This forms a mature ribonuclear complex facilitating normal interaction with the ribosome. If a PTC occurs 50–55 nucleotides upstream of an EJC, this allows the EJC to bind to a kinase-associated protein complex containing the component UF1. Binding triggers release of factors from the complex leading to deadenylation, decapping and exonuclease activity with subsequent degradation of the ribonuclear complex.

If an mRNA transcript containing a premature PTC escapes nonsense-mediated decay, during translation the PTC results in termination of the protein, leading to a truncated protein product. Normal binding of tRNA with its matching site on mRNA takes place within the ribosome 'A' site during translation. A cognate tRNA matches three of its mRNA base pairs, while a near-cognate tRNA matches only two. The normal levels of near-cognate tRNA matches compared to cognate tRNA are less than 0.1% in normal translation [78]. When a PTC exists, no cognate tRNA exists: instead, eukaryotic release factor 1 (eRF1) binds to the stop codon at the 'A' site and initiates release of the polypeptide from the ribosomal complex. Nonsense suppression therapy works by promoting read-through of transcripts with a PTC by promoting binding of near-cognate tRNAs at the PTC instead of eRF1. In clinical usage, aminoglycoside antibiotics exploit this mechanism by binding to bacterial ribosomes and causing fatal translational errors. Eukaryotic cells have greater resistance to the substitution of near-cognate tRNAs due to differences in ribosomal structure. Nonetheless, aminoglycosides such as gentamicin,

paromomycin, streptomycin among others promote read-through at eukaryotic PTC sites. Moosajee et al. showed that in a zebrafish model of choroideremia, where homozygous REP1 knockout caused by *chm*<sup>ru848</sup> usually confers embryonic lethality, administration of gentamicin and paromomycin improved read-through and conferred a 1.5- to 1.7-fold improvement in survival [79].

Use of aminoglycosides is, however, limited by systemic toxicity and retinal toxicity. Synthesis of novel aminoglycosides and aminoglycoside-like molecules has aimed to address this issue, leading to the discovery of a small-molecule PTC124 (Ataluren) which has been shown to increase transcriptional read-through without concomitant toxicity. Delivery of Ataluren topically to the eye has been optimized and it has little retinal toxicity compared with traditional aminoglycosides. Both Ataluren and a similar molecule based on Ataluren, PTC414, has been shown in human *CHM*<sup>Y42X/y</sup> fibroblasts to increase prenylation activity by 45% and 36%: Ataluren has also shown amelioration of ophthalmic and systemic abnormalities in the embryonic *chm*<sup>ru848</sup> zebrafish [80].

Further improvements on efficacy can be achieved by increasing the number of potential transcripts available for read-through promotion. Depending on the position of the PTC, mRNA transcripts may be more or less subject to nonsense-mediated decay, resulting in significant variation of available transcripts between individuals: therefore read-through strategies are highly dependent on mutation location. Downregulating nonsense-mediated decay via inhibition of UF1 leads to an increased number of surviving mRNA transcripts as potential read-through promotion targets.

Nonsense-suppression strategies are theoretically appealing as they can be delivered systemically and non-invasively, and have wide-ranging therapeutic targets beyond the eye. There are currently 34 trials registered for Ataluren (Translarna™) on the Clinical Trials database, including a phase 2 trial for nonsense mutations in aniridia (NCT02647359). Unlike any targeted therapy delivered by subretinal injection, treatment is not limited spatially in the eye. However, previous clinical trials in cystic fibrosis [81] and Duchenne's muscular dystrophy [82] have shown only small treatment effects limited to rigidly defined patient subgroups. By its nature, nonsense-suppression strategies are nonspecific and must promote near-cognate tRNA binding throughout all translation events, potentially increasing production of other anomalous proteins. Furthermore, the applicability of nonsense-suppression strategies may be much narrower than the number of nonsense mutations in *CHM*. Administration of Ataluren to patient-derived fibroblasts carrying a c.772A>T substitution leading to p.Lys258\* failed to show a statistically significant trend in improving prenylation profile compared to non-treated fibroblasts, and did not increase levels of REP1 mRNA or detectable REP1 protein [83]. The authors' *in silico* modeling suggested that the preferred amino acid substitutions provided by Ataluren-induced enhanced read-through were still damaging to REP1 function, and proposed that patients may require mutational screening and modeling before initiation of therapy.

Currently, therefore, while nonsense-suppressing strategies show *in vitro* and some *in vivo* promise, much work remains before it can be a viable therapeutic approach in choroideremia.

## 6. Expert opinion

The first gene therapy treatment for choroideremia was performed in a 57-year-old man 10 years ago in a clinical trial that used AAV2 to deliver the CHM gene by subretinal injection. Since then, the early results have led to a multinational phase III clinical trial involving hundreds of patients across many countries worldwide. During this time, progress has been made with other molecular therapies, such as CRISPR and RNA editing and alternate AAV vectors have also been developed. It is therefore prudent to look back and ask if AAV2 gene replacement is still the preferred option against these developments.

The research so far has a strong emphasis on AAV2-REP1 gene replacement therapy, and with good reason, because it is supported by comprehensive pre-clinical data. Gene augmentation via REP1 replacement has a strong theoretical basis, because virtually all mutations reported to date have been null. REP1 fits well within an AAV vector and preclinical studies have shown strong expression of the transgene within RPE and retina [84]. Though the intervention is invasive and subretinal injection in the degenerate retina of choroideremia is technically challenging, only a single intervention is likely to be required for long-lasting effects. Though there is the theoretical possibility of a mutant truncated protein competing with the transgene product for Rab prenylation, this effect has not been seen *in vitro* [24].

None of the novel evolved AAV capsids to date has shown efficient targeting of the retinal pigment epithelium after intravitreal injection and so the subretinal route of administration is likely to remain the optimal method of gene delivery for the foreseeable future. While the focus on technological innovation over the last 10 years has been on molecular therapies, it should be noted that surgical techniques have also improved considerably during this time and there are now many surgeons with expertise in subretinal gene therapy worldwide.

While base editing and, more pertinently, prime editing have potential applications to a significant number of mutations in choroideremia, an individualized editing approach seems to offer few advantages over gene augmentation in this disease. Although base and prime editing offer unique advantages for correction of the endogenous transcript, these are particularly useful for diseases where the gene length prohibits packaging in an AAV, or where the mutated protein product has a toxic or inhibitory effect. However, this does not appear to be a significant problem in choroideremia.

Two much more interesting approaches with potential are nonsense suppression strategies and antisense oligonucleotides. Nonsense suppression strategies are an extremely appealing idea since they can be delivered orally, they apply to and they are applicable to a large number of mutations in choroideremia. However, clinical trials in other diseases have shown only limited success, they may have more limited applicability than first

appears, and they require long-term administration for effect. Newer formulations of nonsense suppression strategy drugs are more tolerated and have fewer toxic side effects than the drugs from which they are developed, but nonetheless long-term safety data are lacking. However, as companies continue systematically to create and test new molecules, this remains an avenue to watch. Antisense oligonucleotides have shown great promise in LCA with sustained effects, and may be ideal for deep-intronic splice defects. They are delivered by intravitreal injection, which is well tolerated by patients, and retain the gene expression under its endogenous promoter. Unfortunately, however, choroideremia presents few suitable mutation hotspots for targeting using the AON approach. Therefore it is likely that, for the foreseeable future, gene augmentation remains the standard approach to treating choroideremia.

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