Antisense oligonucleotide-based treatment of retinitis pigmentosa caused by USH2A exon 13 mutations

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Mutations in USH2A are among the most common causes of syndromic and non-syndromic retinitis pigmentosa (RP). The two most recurrent mutations in USH2A, c.2299delG and c.2276G > T, both reside in exon 13. Skipping exon 13 from the USH2A transcript presents a potential treatment modality in which the resulting transcript is predicted to encode a slightly shortened usherin protein. Morpholino-induced skipping of ush2a exon 13 in zebrafish ush2armc1 mutants resulted in the production of usherin exon 13 protein and a completely restored retinal function. Antisense oligonucleotides were investigated for their potential to selectively induce human USH2A exon 13 skipping. Lead candidate QR-421a induced a concentration-dependent exon 13 skipping in induced pluripotent stem cell (iPSC)-derived photoreceptor precursors from an Usher syndrome patient homozygous for the c.2299delG mutation. Mouse surrogate mQR-421a reached the retinal outer nuclear layer after a single intravitreal injection and induced a detectable level of exon skipping until at least 6 months post-injection. In conclusion, QR-421a-induced exon skipping proves to be a highly promising treatment option for RP caused by mutations in USH2A exon 13.

INTRODUCTION

Retinitis pigmentosa (RP) is a genetically and clinically heterogeneous disorder characterized by a progressive loss of functional vision caused by the degeneration of the light-sensitive photoreceptor cells in the retina.1 Although being designated as an orphan disease with an overall prevalence of 1–4,000 individuals, RP is the most common type of inherited retinal dystrophy (IRD), affecting ~125,000 patients within the European Union and almost two million individuals worldwide. As such, it imposes a significant burden on health care systems and society in general.

To date, mutations in over 100 genes are known to cause non-syndromic or syndromic RP (https://sph.uth.edu/Retnet/). It is estimated that autosomal recessively inherited RP (arRP) accounts for up to 60% of all RP cases.2 Mutations in USH2A collectively account for 7%–23% of arRP cases and can either result in non-syndromic arRP or in Usher syndrome (combination of RP and hearing impairment).3,4 The mutations in this gene are mostly private and evenly distributed throughout the gene. Three mutations, including c.2299delG, p.(Glu767fs); c.2276G > T, p.(Cys759Phe); and c.7595-2144A > G, p.(Lys2532Thrfs), are derived from a common ancestor and are therefore seen more frequently.1,5–7 The c.2299delG and c.2276G > T mutations represent, respectively, 27.8% and 7.1% of all pathogenic USH2A alleles, and both reside in exon 13 of the USH2A gene.6

Although attempts have been made, clear genotype-phenotype correlations for USH2A mutations have been proven difficult to establish. Generally, nonsense mutations, frameshift mutations, or canonical splice site mutations in USH2A, either biallelic or combined with one missense allele, are associated with Usher syndrome type II, whereas the combination of two missense changes typically results in non-syndromic RP.9 The auditory phenotype of patients with Usher syndrome can be partially compensated by providing patients with hearing aids or cochlear implants.10 However, currently, no

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treatment options exist for the progressive loss of vision associated with mutations in USH2A.

The poor understanding of the physiological role(s) of the usherin protein in photoreceptor cells and the pathophysiological mechanism underlying USH2A-associated RP hampers the development of treatment that interferes with the disease mechanisms. The recent approval of Luxturna (voretigene neparvovec), a gene augmentation therapy for the treatment of patients with RPE65-associated retinal dystrophy,\textsuperscript{11,12} has led to a paradigm shift in the therapeutic approach to monogenic retinal diseases and provides hope for many visually impaired individuals worldwide. However, the development of an USH2A gene augmentation therapy is severely hampered by the size of the usherin-encoding sequence (15,606 nucleotides), which exceeds by a significant margin the cargo capacity of the currently used viral vehicles for gene delivery. An RNA therapy approach, for example, using intravitreal (IVT) delivery of synthetic antisense oligonucleotides (AON) to correct aberrant pre-mRNA splicing, could overcome this limitation for a subset of mutations. In vitro experiments demonstrated that AONs can be used to correct the aberrant pre-mRNA splicing caused by the c.7595-2144A > G mutation in USH2A transcript.\textsuperscript{7} The promise for clinical application of AON-USH2A, pre-mRNA splicing caused by the c.7595-2144A > G mutation in overcomes this limitation for a subset of mutations.\textsuperscript{7} The promise for clinical application of AON-USH2A, pre-mRNA splicing caused by the c.7595-2144A > G mutation in

To validate exon 13 skipping as a potential therapeutic strategy, we employed our previously characterized ush2a zebrafish mutant (ush2a\textsuperscript{mrc1}) that contains a frameshift-inducing mutation in exon 13.\textsuperscript{14} We previously reported that the electroretinogram (ERG) response is significantly reduced in homozygous ush2a\textsuperscript{mrc1} larvae and that the usherin protein is absent from the retina, indicating that ush2a\textsuperscript{mrc1} is a true null allele. The length of USH2A exon 13 is well conserved between human (642 nucleotides) and zebrafish (648 nucleotides), and the (spacing between) cysteine residues that are essential for EGF-lam domain formation are identical (Figure 2A). Following the previously published guidelines for AON design,\textsuperscript{19,20} six antisense phosphorodiامidate morpholino oligomers (PMOs) were designed to target the zebrafish ush2a exon 13 splice acceptor site, splice donor site, or exonic splice enhancer (ESE) motifs (Figure S1A; Table 1). The exon-skipping potential of the PMOs was first investigated by injecting the individual PMOs into the yolk of 1- to 2-cell-stage ush2a\textsuperscript{mrc1} embryos (Figure S1B). Combined delivery of a low dose of two of the most potent PMOs, targeting different regions of ush2a exon 13, resulted in a more efficient skipping of ush2a exon 13 than individually injected PMOs (Figures S2A and S2B). The combination of PMO1 and PMO2 appeared most potent after reverse transcripase (RT)-PCR analysis, without leading to aberrations in overall body morphology, and was subsequently used to determine whether exon 13 skipping had an effect on the phenotypic outcome of the ush2a\textsuperscript{mrc1} mutant (Figures 2B and S2C).

We first determined whether skipping of zebrafish ush2a exon 13 in homozygous ush2a\textsuperscript{mrc1} mutant larvae resulted in the synthesis of a shortened usherin protein (usherin\textsubscript{exon 13}). Antibodies directed against the intracellular region of zebrafish usherin were used to stain fixed cryosections of wild-type larvae, uninjected ush2a\textsuperscript{mrc1} larvae, and ush2a\textsuperscript{mrc1} larvae in which ush2a exon 13 skipping was induced by PMO injection (Figure 2C, green signal). The photoreceptor connecting cilium was labeled by antibodies against centrin (Figure 2C, red

In this study, we explored AON-induced exon skipping as a potential treatment modality for patients with RP caused by mutations in exon 13 of the USH2A gene. As this exon consists of a multiplier of three nucleotides, skipping the exon does not disturb the open reading frame and could result in the production of a shortened protein with predicted residual function. With the use of our previously characterized ush2a\textsuperscript{mrc1} zebrafish model,\textsuperscript{14} we demonstrate that usherin lacking the amino acids encoded by exon 13 has sufficient residual function to prevent loss of retinal function. With the use of cellular models, we identified and validated AON QR-421a as candidate molecule for an exon-skipping therapy described above. Currently, QR-421a is being evaluated in the phase 1/2 clinical trial (Clinicaltrials.gov: NCT03780257).

**RESULTS**

**Formation of epidermal growth factor (EGF)-like fusion domain after targeted USH2A exon 13 skipping**

Wild-type usherin is predicted to contain ten EGF-lam domains (http://smart.embl-heidelberg.de/). EGF-lam domains typically contain eight cysteine residues that interact in a pairwise fashion, through covalent disulfide bond, necessary for protein folding and stability. These EGF-lam domains in usherin harbor multiple protein-truncating mutations. Also, 22 out of the 80 cysteine residues in these EGF-lam domains have been found to be mutated in patients with USH2A-associated RP (USH2A LOVD mutation database, https://www.lovd.nl/USH2A), five of which reside within the protein region encoded by exon 13. Unpaired cysteine residues contain a reactive-free thiol group that can induce unwanted multimerization or crosslinking with other proteins.\textsuperscript{15,16} The in-frame skipping of exon 13 is predicted to result in the fusion of parts of EGF-lam domains 4 and 8 into a functionally related EGF-like domain (Figure 1A). EGF-like domains contain six cysteine residues that together create three disulfide bonds by cysteines 1 + 3, 2 + 4, and 5 + 8 in the fused EGF-like-domain.\textsuperscript{17} There are 16 amino acids between the fifth and sixth cysteine residue in the EGF-like 4-8 fusion domain, which is different from the canonical spacing between cysteine residue 5 and 6 within EGF-like domains, namely 8 amino acids.\textsuperscript{18} However, 3D homology modeling predicted normal disulfide bonds by cysteines 1 + 3, 2 + 4, and 5 + 8 in the fused EGF-like-domain.\textsuperscript{17} There are 16 amino acids between the fifth and sixth cysteine residue in the EGF-like 4-8 fusion domain, which is different from the canonical spacing between cysteine residue 5 and 6 within EGF-like domains, namely 8 amino acids.\textsuperscript{18} However, 3D homology modeling predicted normal disulfide bridge formation within the EGF-like 4-8 fusion domain (Figure 1B). In conclusion, molecular modeling warrants exploring the effect of exon 13 skipping at the level of visual function.

**AON-induced skipping of ush2a exon 13 in a mutant zebrafish model restores usherin protein expression and visual function**

To validate exon 13 skipping as a potential therapeutic strategy, we employed our previously characterized ush2a zebrafish mutant (ush2a\textsuperscript{mrc1}) that contains a frameshift-inducing mutation in exon 13.\textsuperscript{14} We previously reported that the electroretinogram (ERG) response is significantly reduced in homozygous ush2a\textsuperscript{mrc1} larvae and that the usherin protein is absent from the retina, indicating that ush2a\textsuperscript{mrc1} is a true null allele. The length of USH2A exon 13 is well conserved between human (642 nucleotides) and zebrafish (648 nucleotides), and the (spacing between) cysteine residues that are essential for EGF-lam domain formation are identical (Figure 2A). Following the previously published guidelines for AON design,\textsuperscript{19,20} six antisense phosphorodiамidate morpholino oligomers (PMOs) were designed to target the zebrafish ush2a exon 13 splice acceptor site, splice donor site, or exonic splice enhancer (ESE) motifs (Figure S1A; Table 1). The exon-skipping potential of the PMOs was first investigated by injecting the individual PMOs into the yolk of 1- to 2-cell-stage ush2a\textsuperscript{mrc1} embryos (Figure S1B). Combined delivery of a low dose of two of the most potent PMOs, targeting different regions of ush2a exon 13, resulted in a more efficient skipping of ush2a exon 13 than individually injected PMOs (Figures S2A and S2B). The combination of PMO1 and PMO2 appeared most potent after reverse transcripase (RT)-PCR analysis, without leading to aberrations in overall body morphology, and was subsequently used to determine whether exon 13 skipping had an effect on the phenotypic outcome of the ush2a\textsuperscript{mrc1} mutant (Figures 2B and S2C).
ERGs were subsequently recorded from zebrafish larvae. This corroborated that exon 13 skipping resulted in the synthesis of usherin transcripts as compared to uninjected larvae from the same clutch (34.98 ± 0.0001 [Kruskal-Wallis and Dunn test] and p < 0.001 [control PMO-injected]; Kruskal-Wallis and Dunn test) versus age- and strain-matched wild-type larvae (p < 0.05 [uninjected]; n = 10; p < 0.0001 [Kruskal-Wallis and Dunn’s nonparametric test]) (Figure 2D). This corroborated that exon 13 skipping resulted in the synthesis of usherinΔexon 13.

ERGs were subsequently recorded from ush2a rmcl larvae that were injected with a combination of ush2a exon 13-targeting PMOs (n = 25) or with a standard control PMO (n = 14). Uninjected age- and strain-matched wild-types (n = 10) and ush2a rmcl (n = 11) larvae were used as controls. Uninjected and control PMO-injected ush2a rmcl mutant larvae demonstrated significantly reduced b-wave amplitudes as compared to age- and strain-matched wild-type larvae (p < 0.05 [uninjected], and p < 0.001 [control PMO-injected]; Kruskal-Wallis and Dunn’s nonparametric test) (Figures 2E and 2F). PMO-induced skipping of ush2a exon 13 from ush2a rmcl larvae resulted in significantly increased b-wave amplitudes as compared to uninjected or control PMO-injected ush2a rmcl larvae, which is indicative for a restoration of visual function. The ERG b-wave amplitudes recorded in ush2a rmcl larvae after injection with exon 13-targeting PMOs were not significantly different from those recorded in age- and strain-matched wild-type larvae (p > 0.999) (Figures 2E and 2F). Quantitative RT-PCR (qRT-PCR) analysis of exon 13 skipping in larvae injected with low or high doses of PMOs revealed that increasing the PMO dose did not result in a significant gain in ush2a Δexon 13 transcripts but rather decreased the number of full-length ush2a transcripts (Figures 2G and S2D). At all tested doses of PMO, the levels of ush2a Δexon 13 transcripts ranged between 18% and 26% of the amount of total ush2a transcripts observed in wild-type zebrafish. Together, these data show that AON-induced skipping results in the formation and correct localization of an usherinΔexon 13 protein with sufficient residual function to rescue visual dysfunction in ush2a rmcl zebrafish larvae.

**Identification of lead oligonucleotide QR-421a**

Based on the ability of usherinΔexon 13 to restore visual function in zebrafish, we aimed to develop AONs with the ability to induce skipping of exon 13 from human USH2A transcripts. Fourteen AONs were designed based on the bio-informatic analysis of the sequence of USH2A exon 13 and flanking intronic regions. Both the intron-exon boundaries and the ESE motifs within exon 13, identified using the SpliceAid webserver, were used as targets for AONs. With the use of in silico analysis, parameters for (lack of) secondary structure formation, thermodynamic properties, and sequence selectivity were taken into account to minimize potential off-target effects. The designed AONs were transfected in the retinoblastoma-derived WERI-Rb1 cell line at a concentration of 200 nM and screened for their potential to induce USH2A exon 13 skipping (Figure S3). Because of these analyses, the
Figure 2. Morpholino antisense oligonucleotides (AONs) mediate ush2a exon 13 skipping, usherin exon 13 protein expression, and restoration of electroretinogram (ERG) in a mutant zebrafish model

(A) Amino acid alignment of the sequences encoded by human and zebrafish USH2A exon 13. The (partial) EGF-lam domains are indicated. The cysteine residues required for 3D topology of the EGF-lam domains (green) are completely conserved between zebrafish and human. (B) Phosphorodiamidate morpholino oligonucleotide (PMO)-induced skipping of ush2a exon 13 in zebrafish larvae. ush2armc1 mutant embryos were injected with a combination of PMO1 and PMO2 (1 ng of each). Investigation of ush2a pre-mRNA splicing at 3 days post-fertilization (dpf) revealed the skipping of ush2a exon 13 upon injection of PMOs targeting ush2a exon 13. Uninjected ush2armc1 mutant zebrafish larvae and WT larvae were used as controls. (C) Subcellular localization of usherin in horizontal cryosections of larval (5 dpf) zebrafish retinae. Usherin was visualized with anti-usherin antibodies directed against the intracellular C-terminal tail of zebrafish usherin (green signal). Nuclei were stained with DAPI (blue signal), and the connecting cilium is labeled using anti-centrin antibodies (red). In WT larvae, usherin is present at the photoreceptor periciliary membrane, adjacent to the connecting cilium. In homozygous ush2armc1 larvae, no specific usherin signal could be detected. PMO-induced ush2a exon 13 skipping in ush2armc1 mutant larvae resulted in partial restoration of usherin exon 13 expression with the correct subcellular localization in the retina. OS, outer segment; ONL, outer nuclear layer; OPL, outer plexiform layer; IPL, inner plexiform layer; wt: WT; ush2armc1, zebrafish with exon 13 mutation. (D) Scatterplot of the relative fluorescence intensity of anti-usherin staining in the periciliary region of all photoreceptors in the middle section of the larval zebrafish eye. The signal intensity is decreased in the ush2armc1 retina compared to WTs. Relative fluorescent signal intensity of anti-usherin staining is significantly increased in PMO-injected ush2armc1-children as compared to uninjected or control PMO-injected mutants (****p < 0.0001, Kruskal-Wallis test followed by Dunn’s nonparametric post-test). (E) Average ERG b-wave traces from uninjected, control PMO-injected, exon 13 PMO-injected ush2armc1 larvae and WT controls at 5–6 dpf. PMO-induced skipping of ush2a exon 13 completely restored b-wave amplitudes in ush2armc1 larvae as compared to uninjected or control PMO-injected mutants. (F) Maximum b-
best-performing 21-mer RNA AON sequence was selected. For further preclinical development, the molecule was synthesized as an antisense RNA molecule with 2′-O-(2-methoxyethyl) ribose sugar modification and a fully phosphorothioated backbone. This candidate was named QR-421a thereafter.

QR-421a was screened for pro-inflammatory potential in silico, revealing the absence of known inflammatory motifs, and in vitro, using a human peripheral blood mononuclear cell (PBMC) activation assay. Gymnotic delivery of QR-421a at concentrations between 0.1 and 10 μM had no effect on PBMC viability and no statistically significant increase in cytokine release (Figure S4).

The target specificity of QR-421a was investigated using an in silico analysis. QR-421a showed no full complementarity to any mRNA, pre-mRNA, or DNA targets other than the anticipated region in USH2A. Partial complementarity to other genomic regions was only found with ≥ 2 mismatches. Only two off-target sequences were identified with 2 mismatches, residing in one intergenic and one intronic region, and are therefore not expected to influence gene expression or pre-mRNA splicing. Other hits with > 2 mismatches are not considered biologically meaningful for a 21-mer splice modulation oligonucleotide, as a single mismatch in an AON was previously shown to already markedly decrease splice modulation efficiency.23 Hence, the risk for potential off-target splicing effects, due to the hybridization of QR-421a to targets other than the intended target, is considered negligible.

WERI-Rb1 cells were treated with QR-421a gymnotically or with the aid of a transfection reagent to provide pharmacodynamic proof of concept for the USH2A exon 13 skipping potential using transcript-specific qRT-digital droplet PCR (ddPCR) analysis. Upon QR-421a transfection, a dose-dependent skipping of USH2A exon 13 was induced, which was already evident at a concentration of 25 nM. An exon 13 skipping efficiency of ~60% was reached upon transfection at the highest concentration tested (200 nM) (Figure 3A). After gymnotic delivery of QR-421a at concentrations ranging from 0 to 50 μM, exon 13 skipping efficiencies ranging from 10% to 17% were observed (Figure 3B). In both experiments, treatment of WERI-Rb1 cells with a control oligonucleotide did not induce skipping of exon 13, confirming that the observed exon-skipping potential is specific for QR-421a (Figures 3A and 3B). Amplification of USH2A exons 11 to 15 in QR-421a-treated WERI-Rb1 cells (200 nM) revealed mainly transcripts lacking exon 13, which was confirmed by Sanger sequencing but also two minor alternative products that were also identified in untreated WERI-Rb1 cells (Figures 3C and 3D). One of these fragments lacked both exons 12 and 13; the other fragment contained only exons 11 and 15. Altogether, these data show that QR-421a has the ability to enter proliferating WERI-Rb1 cells after transfection or even unaided, thereby inducing a concentration-dependent skipping of USH2A exon 13.

QR-421a treatment induces a concentration-dependent increase of USH2A exon 13 skipping in induced pluripotent stem cell (iPSC)-derived photoreceptor progenitor cells (PPCs) PPCs, differentiated from iPSCs obtained from an USH2A patient with a homozygous c.2299delG mutation in exon 13, were used to assess the exon-skipping potential of QR-421a in a differentiated cell model with the appropriate genetic context. PPCs have been previously shown to be a valuable and clinically relevant tool for the evaluation of novel human-specific therapeutic strategies.24,25 Initially, patient-derived fibroblasts were reprogrammed into iPSCs and subsequently differentiated into PPCs. In order to validate that the cells had differentiated into PPCs, we assessed the expression levels of photoreceptor marker genes (CRX, NRL, OPN1SW, OPN1LW, and RHO) by qRT-PCR analysis after 90 days of differentiation. As expected, the expression levels of photoreceptor marker genes were all significantly increased as compared to iPSCs, whereas the expression of the iPSC-specific marker gene NANOG was simultaneously decreased (Figure 4A).

Patient-derived PPCs were treated with a stable concentration of QR-421a for 28 days using gymnotic delivery. Every 2 days, one-half of the culture medium was replaced with fresh medium containing a new dose of QR-421a. Untreated PPCs and PPCs treated with a control oligonucleotide (with the same chemistry and length but a random sequence) were used as negative controls. RT-PCR analysis of USH2A exons 11 to 15 revealed that, in contrast to previous analysis in patient-derived fibroblasts,26 no alternatively spliced USH2A transcripts could be detected in untreated PPCs homozygous for the c.2299delG mutation (Figure 4B). Results furthermore showed that QR-421a induced significant levels of exon 13 skipping at all concentrations tested (1–10 μM), whereas exons 12 and 14 were retained within the USH2A Δexon 13 transcript (Figures 4B and 4C). At a 1-μM concentration, exon 13 skipping was observed in 42% ± 11% (p = 0.001, Sidak’s multiple comparison test) of USH2A transcripts. This increased to 63% ± 8% (p < 0.0001, Sidak’s multiple comparison test) of transcripts lacking exon 13 when QR-421a was supplied at a 10-μM concentration (Figure 4C). No exon 13 skipping was detected in untreated or control oligonucleotide-treated PPCs, indicating that skipping of this exon was specifically induced by QR-421a.

Retinal uptake, efficacy, and duration of action of mQR-421a in wild-type mouse retina In the absence of a humanized exon 13 mutant mouse model, wild-type mice were explored as a model system to study the molecular in vivo efficacy of QR-421a. The USH2A gene is well conserved across...
species and is very similar in humans and mice. However, the mouse sequence has a few base differences at the QR-421a binding site; therefore, a mouse surrogate mQR-421a was used in the mouse studies. mQR-421a has the same chemistry, length, binding site, and sequence as QR-421a, the only difference being four bases that are changed to methoxyethyl RNA bases.

Next, levels of Ush2a transcripts with and without exon 12 were quantified using isoform-specific RT-ddPCR assays, and the percentage of exon skipping was calculated. Results showed that mQR-421a induced Ush2a exon 12 skipping at all of the tested doses compared to the control AON (Figure 5C). Exon-skipping levels were dose dependent and ranged from 12% at the 7.5-μg dose to 29% at the 60-μg dose. In comparison, only <1.5% exon skipping was detected in the control AON-treated group.

To study the duration of action in vivo, mice received bilateral IVT injections of 30 μg mQR-421a per eye in a single dosing occasion and were maintained for 1, 2, 14, 28, 56, 103, or 203 days. Levels of Ush2a transcripts with and without exon 12 were quantified using isoform-specific RT-ddPCR assays, and the percentage of exon skipping was calculated. Results showed that mQR-421a induced significant levels of Ush2a exon 12 skipping at all time points tested in this study (Figure 5D). Skipping levels increased with time, and highest skipping was detected at 56 days, and the skipping levels decreased slightly thereafter. An average of 25% and 20% exon skipping was detected, respectively, at 1 and 2 days post-dose. Exon skipping increased over time to approximately 40%, 50%, and 53% at 17, 28, and 56 days post-dose, respectively. At days 103 and 203 post-dose, skipping percentage decreased to 43% and 38%, respectively.

**DISCUSSION**

Mutations in exon 13 of the USH2A gene, including the recurrent mutations c.2299delG and c.2276G > T, are estimated to underlie syndromic (Usher syndrome) and non-syndromic RP in approximately 16,000 individuals in the Western world. In this study, we used PMOs targeting zebrafish ush2a exon 13 to evaluate exon skipping as a therapeutic strategy for the future treatment of USH2A-associated RP. We show that skipping of ush2a exon 13 resulted in a 11 + 12 skipping, indicating the natural skipping of these exons, albeit at very low levels. Next, levels of Ush2a transcripts with and without exon 12 were quantified using isoform-specific RT-ddPCR assays, and the percentage of exon skipping was calculated. Results showed that mQR-421a induced Ush2a exon 12 skipping at all of the tested doses compared to the control AON (Figure 5C). Exon-skipping levels were dose dependent and ranged from 12% at the 7.5-μg dose to 29% at the 60-μg dose. In comparison, only <1.5% exon skipping was detected in the control AON-treated group. Doses higher than 60 μg did not result in an increase in exon skip; rather, a slight reduction was noticed. To study the duration of action in vivo, mice received bilateral IVT injections of 30 μg mQR-421a per eye in a single dosing occasion and were maintained for 1, 2, 14, 28, 56, 103, or 203 days. Levels of Ush2a transcripts with and without exon 12 were quantified using isoform-specific RT-ddPCR assays, and the percentage of exon skipping was calculated. Results showed that mQR-421a induced significant levels of Ush2a exon 12 skipping at all time points tested in this study (Figure 5D). Skipping levels increased with time, and highest skipping was detected at 56 days, and the skipping levels decreased slightly thereafter. An average of 25% and 20% exon skipping was detected, respectively, at 1 and 2 days post-dose. Exon skipping increased over time to approximately 40%, 50%, and 53% at 17, 28, and 56 days post-dose, respectively. At days 103 and 203 post-dose, skipping percentage decreased to 43% and 38%, respectively.

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partially restored expression of usherin protein in photoreceptors of ush2armc1 larvae. Furthermore, exon 13 skipping restored ERG b-wave amplitudes, which indicates improved retinal function. We established QR-421a, an AON drug candidate that induces skipping of human USH2A exon 13 in cellular models. Finally, the corresponding mouse AON showed a long in vivo duration of action in mouse retina following a single IVT treatment. Our study therefore provides proof of concept for exon skipping as a highly promising treatment option for USH2A-associated retinal degeneration as a consequence of mutations in exon 13.

Figure 3. QR-421a shows a concentration-dependent increase of USH2A exon 13 skipping in WERI-Rb1 cells
(A and B) WERI-Rb1 retinoblastoma cells were treated with different concentrations of QR-421a, using either (A) transfection or (B) gymnotic uptake. Untreated and control oligo-treated cells were included as negative controls. Exon-skipping level was determined by quantification of USH2A transcripts with and without exon 13 by RT-ddPCR. Treatment with QR-421a resulted in a significant concentration-dependent increase of USH2A Δexon 13 transcripts. Data are shown as mean ± SD. Two biological replicates per treatment condition. Asterisks indicate significant differences with scrambled control oligo-treated cells (**p < 0.001; ****p < 0.0001; one-way ANOVA followed by Dunnett’s multiple comparison test).
(C) Representative image of exon 11-15 RT-PCR amplifiers obtained with RNA isolated from untransfected and QR-421a-transfected WERI-Rb1 cells. QR-421a is able to induce skipping of USH2A exon 13 and does not increase the formation of other alternatively spliced USH2A transcripts. Of note, USH2A Δexon 12-13 and Δexon 12-14 transcripts are already present in untreated WERI-Rb1 cells and yield out-of-frame mRNA transcripts. (D) Sanger sequencing traces of the USH2A Δexon 13 amplicon shown in (C) confirm that the sequence of exon 13 is lacking from the transcript.

The in-frame skipping of exons harboring pathogenic mutations has already been shown to have a particularly high therapeutic potential for large genes encoding (structural) proteins that contain a series of repetitive protein domains. Duchenne muscular dystrophy (DMD) is caused by mutations in the DMD gene. DMD encodes dystrophin, a structural linker protein consisting of a stretch of 24 spectrin-like domains flanked by protein-protein interaction domains that are used to connect the F-actin cytoskeleton to β-dystroglycan. Exon skipping was previously shown to restore the reading frame in patients suffering from DMD due to mutations in exon 51 and has the ability to restore the production of a functional dystrophin protein. Like dystrophin, usherin is also a large structural protein and contains repetitive EGF-lam and FN3 domains. The in-frame exon 13 of USH2A, containing the recurring mutations c.2299delG and c.2276G > T, encodes multiple EGF-lam domains that are proposed to form a stiff rod-like element.

In the inner ear, usherin is essential for the maturation of hair bundles that are located at the apex of hair cells, whereas in the retina, the large extracellular tails of usherin and ADGRV1 have been proposed to interact and together, bridge the gap between the opposing membranes of the photoreceptor connecting cilium and the periciliary region. In contrast to the situation in the inner-ear hair cells, usherin seems redundant for the initial development of photoreceptors and rather fulfills a post-developmental role. As such, usherin seems to be particularly important for photoreceptor maintenance. Therefore, therapeutic strategies that rescue the expression of functional usherin protein in the retina can potentially prevent or slow down the progression of photoreceptor degeneration and, as such, preserve visual function in patients.
transcripts results in a shortened usherin protein that is able to properly localize in zebrafish photoreceptor cells. Furthermore, PMO-induced skipping of the mutated exon 13 resulted in completely restored ERG b-wave amplitudes, indicative of a restored visual function. Although PMOs are remarkably stable in zebrafish embryos, the intranuclear PMO concentration declines with the increasing number of nuclei during development. As such, the effect of PMO-induced intranuclear PMO concentration declines with the increasing number of nuclei during development. As such, the effect of PMO-induced intranuclear PMO concentration declines with the increasing number of nuclei during development. As such, the effect of PMO-induced intranuclear PMO concentration declines with the increasing number of nuclei during development. As such, the effect of PMO-induced intranuclear PMO concentration declines with the increasing number of nuclei during development. As such, the effect of PMO-induced intranuclear PMO concentration declines with the increasing number of nuclei during development.

The observed levels of exon 13 skipping (~20% at 3 days post-fertilization [dpf]) already indicate that relatively few *ush2a Δ*exon 13 transcripts are required to rescue the retinal defects in *ush2a d*mc1 larvae. As it is only useful to record ERGs in zebrafish larvae that have a functional retina (≥5 dpf),25 the complete restoration of ERG defects observed in PMO-treated *ush2a d*mc1 larvae, at 5–6 dpf, either suggests that even lower levels of *ush2a Δ*exon 13 transcripts are sufficient for retinal function or that the encoded usherinΔexon 13 is relatively stable, at least until 5–6 dpf. Rods do not significantly contribute to the zebrafish ERG until 15 dpf, and therefore, all responses recorded in these larvae are expected to be cone derived.39,40 Patients with *USH2A*-associated RP often present with night blindness as the initial symptom of retinal dysfunction, indicating a primary dysfunction of the rods.61 However, it was recently reported that both rod and cone responses were markedly reduced in the ERGs of adolescent USH2a patients.62 Therefore, a restored ERG response in zebrafish *ush2a d*mc1 larvae upon exon 13 skipping is promising for a beneficial effect in patients. Furthermore, the functionality of the usherinΔexon 13 protein is also supported by a recent book chapter by Pendse et al.43, demonstrating that auditory function is not affected in *Ush2aΔ*exon 13/mut mice.

Following the therapeutic proof of concept obtained for *ush2a* exon 13 skipping in zebrafish photoreceptors, we present evidence for the pharmacodynamic potential of QR-421a, the lead-candidate AON for the future treatment of patients with RP due to mutations in exon 13 of the *USH2A* gene. QR-421a treatment resulted in a concentration-dependent *USH2A* exon 13 skipping in a retinoblastoma cell line. In general, retinal tissue displays a high degree of transcriptional activity and alternative splicing.44 Retinal organoids and PPC differentiated from patient-derived iPSCs provide an excellent platform to test therapeutic interventions for IRDs in *vitro*, as recently demonstrated by us62 and others.27 Treatment of PPCs, derived from a patient homzygous for the *USH2A c.2299delG* mutation with QR-421a, resulted in a dose-dependent skipping of exon 13, with no induction of unwanted, alternative exon-skipping events observed.

It is important that oligonucleotides intended for the treatment of retinal dystrophies are capable of accessing retinal cells in order to reach the intended target site. In this study, *in vivo* efficacy of a mouse surrogate, mQR-421a, was characterized in wild-type mice following IVT administration of the AON. Upon IVT dose, mQR-421a was...
A maximum percentage of ~20% of ush2a Δexon 13 transcripts was observed in PMO-treated ush2a rm2 zebrafish larvae relative to the total amount of ush2a transcripts in untreated strain- and age-matched wild-type larvae. As this relatively low percentage of exon 13 skipping still resulted in a complete restoration of ERG traces, it is tempting to speculate on the minimal amount of USH2A Δexon 13 transcripts needed for a detectable and durable therapeutic effect. Individuals that carry a heterozygous loss-of-function mutation in USH2A are asymptomatic, indicating that about 50% of wild-type usherin would never result in sufficient protein restoration to rescue auditory function in mice. The surprisingly low amount of usherinΔexon 13 protein detected in the retina of PMO-injected ush2armc1 larvae, nevertheless leading to complete restoration of retinal function, suggests that an even lower amount of usherinΔexon 13 protein can be sufficient for retinal function. Interestingly, studies in a humanized mouse model for USH1c showed that ~20% of correctly spliced Ush1c transcripts, observed after the delivery of splice-correcting AONs at postnatal day 5, is sufficient to rescue auditory function up to 3 months post-injection. Based on this and what is known from other AONs acting through an exon-skipping mechanism,16–18 exon-skipping levels in the range of 10%–20% could potentially be enough to result in sufficient protein restoration to reach efficacious levels.

The age of onset and slow rate of progression of USH2A-associated RP leave ample opportunity for therapeutic intervention to halt the
slow disease progression. All evidence indicates that ush2a transcripts lacking exon 13 encode a usherinΔexon 13 protein with sufficient residual function to rescue visual function. The splice-modulating functionality of QR-421a was demonstrated in vitro and in vivo for the treatment of RP, resulting from mutations in exon 13 of the USH2A gene. QR-421a is currently being investigated in a phase I/II clinical trial (ClinicalTrials.gov: NCT03780257), and to our knowledge, this is the first time that proof of concept for a molecular treatment that went into clinical trials was solely obtained in zebrafish, stressing the value of this model organism in translational science.

MATERIALS AND METHODS

Animals

ush2a<sup>mel</sup> (c.2337_2342delinsAC; p.Cys780GlnfsTer32; Dona et al.14) and strain-matched wild-type Tüpfel long fin zebrafish were bred and raised under standard conditions.69 Both adult and larval zebrafish were kept at a light-dark regime of 14 h light:10 h darkness. All experiments were carried out in accordance with European guidelines on animal experiments (2010/63/EU). Zebrafish eggs were obtained from natural spawning and reared at 28.5°C in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub>, and 0.33 mM MgSO<sub>4</sub>), supplemented with 0.1% methylene blue. Wild-type male and female C57BL/6J mice between 9 and 13 weeks (at start of treatment) were randomly assigned to different groups, with balanced age and gender distribution. Sample sizes were estimated based on power analysis. Male and female C57BL/6J mice between 9 and 13 weeks (at start of treatment) were randomly assigned to different groups, with balanced age and gender distribution. Sample sizes were estimated based on power analysis. Mice were group housed under pathogen-free conditions with ad libitum access to water and food. Mouse experiments were approved by the local Ethics Committee and conformed to the European Community regulations (EEC number 86/609).

In silico modeling of the effect of USH2A exon 13 skipping on the EGF-lam protein domain structure

Models of the separate EGF domains were built using the modeling script in the YASARA<sup>50</sup> and WHAT IF<sup>51</sup> algorithms with standard parameters. The separate domain sequences were used, resulting in a model for each domain based on PDB: 4AQS (domain 4, 45% sequence identity), 3TBD (domain 5, 46% sequence identity), 4AQS (domain 6, 44% sequence identity), 5LF2 (domain 7, 51% sequence identity), and 1KLO (domain 8, 41% sequence identity) (https://www.rcsb.org/). To create the fusion model of domains 4 and 8, we used the model for domain 4 as template and swapped the C-terminal residues for their corresponding residues in domain 8. A subsequent energy minimization was performed to remove big errors.

Zebrafish PMO design and microinjection

PMOs were designed by first assessing the target sequence for SRSF2 (SC35) ESE sites (threshold of 3.0) using the online ESE finder 3.0 tool.<sup>52</sup> Zebrafish ush2a exon 13-targeting PMOs were synthesized by Gene Tools (USA). PMOs were dissolved in ultrapure water at a stock concentration of 50 μg/μL and stored at −20°C. One nanoliter containing 0.5—4 pg per PMO and 0.25% (v/v) phenol red was injected into the yolk of 1- to 2-cell-stage embryos with a Pneumatic Picopump pv280 (World Precision Instruments). After injection, embryos were raised at 28.5°C in E3 embryo medium until analysis. PMO sequences are provided in Table 1.

Zebrafish ush2a transcript analysis

Total RNA was isolated from pools of 10—15 larvae per condition. Larvae were snap frozen on liquid nitrogen and subsequently homogenized in QIAzol reagent (QIAGEN; #79306) using a 25G needle. QIAzol extraction of total RNA was performed as per the manufacturer’s instruction. Total RNA was DNase treated using the NucleoSpin RNA Extraction Kit (Macherey-Nagel; #740955.50). 1 μg of total RNA was reverse transcribed using the SuperScript VILO Reverse Transcriptase Kit (Thermo Fisher Scientific; #11755050). PMO-induced alternative splicing of ush2a transcripts was analyzed by PCR amplification using primers in zebrafish ush2a exons 11 and 14 using Q5 HF DNA polymerase (New England Biolabs; #M0491L). Primer sequences are provided in Table 2. Amplified transcripts were visualized by agarose gel electrophoresis (1% agarose in 0.5 × Tris-borate-EDTA [TBE]) and subsequently validated using Sanger sequencing. Exon 13-skipping levels were determined using a qRT-PCR approach, including a standard curve of custom synthetic oligonucleotide templates (gBlocks; Integrated DNA Technologies), using primer pairs that specifically amplify exon 13-containing ush2a transcripts or Δexon 13 ush2a transcripts. Targets were amplified using GoTaq DNA polymerase (Promega; #M3001) and analyzed on a QuantStudio 3 Real-Time PCR System (Applied Biosystems).

Zebrafish immunohistochemistry and quantification of fluorescent signal intensity

Per group, 10 zebrafish larvae were imbedded in Tissue-Tek O.C.T. (Optimal Cutting Temperature) compound (Sakura; #4583) without prior fixation. Unfixed cryosections were permeabilized using 0.01% Tween 20 (Merck; #8.22184) in PBS, rinsed, and then pre-incubated with blocking solution (1% bovine serum albumin; Sigma-Aldrich; #A9617) in PBS for 1 h at room temperature. After rinsing the sections three times with PBS, they were incubated for 1 h at room temperature with blocking solution containing the secondary antibodies (Alexa Fluor 488 goat anti-rabbit [1:200; Molecular Probes; #A11008] and Alexa Fluor 568 goat anti-mouse [1:200; molecular Probes; #A11011]) and the nuclei staining 4',6-diamidino-2-phenylindole (DAPI; diluted 1:8000; Molecular Probes; #D1306). After a dip in ultrapure water, the sections were coveredslipped with ProLong Gold Antifade Reagent (Life Technologies; #P39930; lot. #173258). The sections were examined using a Zeiss Axio Imager Z2 microscope with ZEN 2012 software and photographed with a Zeiss Axiocam 506 mono camera. Images of the middle section of each eye, taken at identical exposure settings, were used for quantification of the fluorescent signal intensity of anti-usherin immunoreactivity using the Fiji...
to identify the usherin immunofluorescence, the centrin mask and layer containing the usherin immunofluorescence, the exact location of usherin immunofluorescence within the centrin mask. The resulting mask was dilated three times, and touching objects were separated using the watershed option. Subsequently, the maximum gray value of the identified regions was measured on the original image of usherin immunofluorescence ("analyse particles" option; size = 0–50, pixel circularity = 0.00–1.00).

Table 2. Primer/probe list

| Target       | Species            | Sequence                                      |
|--------------|--------------------|-----------------------------------------------|
| ush2a exon 11-14 | zebrafish           | 5′-AGGCTGTCGAGTCTCTTC-3′                      |
| ush2a exon 12 + 13 | zebrafish           | 5′-TGTATCTGCTTACCCACACG-3′                   |
| Δexon 13 ush2a | zebrafish           | 5′-AGTTGACATCACGTCACCTG-3′                   |
| USH2A exon 11-15 | human              | 5′-CGGACAGGAAAACCCAGTAC-3′                   |
| NANOG        | human              | 5′-AGGCTGTCGAGTCTCTTC-3′                      |
| CRX          | human              | 5′-GCCCTATGGTATGGGCTTG-3′                    |
| NRL          | human              | 5′-GCAGCTTGCAGTACCCACACG-3′                  |
| OPN1SW       | human              | 5′-ACCTATGGATGGGGCTTG-3′                     |
| OPN1LW       | human              | 5′-GAGAGAGGCAATGAAGC-3′                      |
| USH2A Δexon 13 | human              | 5′-GAGGAGGCAATGAAGC-3′                       |
| KHO          | human              | 5′-CTGTTCTGCTGCTGCT-3′                       |
| GUS8         | human              | 5′-GCAGCTTGCAGTACCCACACG-3′                  |
| USH2A exon 11-15 | human              | 5′-AGTTGACATCACGTCACCTG-3′                   |
| USH2A Δexon 13 | human              | 5′-AGTTGACATCACGTCACCTG-3′                   |
| Ush2a exon 10-14 | mouse             | 5′-TTACCGACCTTTGGTTGTCG-3′                   |
| Ush2a Δexon 12 | mouse             | 5′-CCATCTGCGGCTGCTCGTAC-3′                  |

ERG recordings in zebrafish larvae

Larvae, of 5–6 dpf, were placed on a filter paper in the middle of a plastic recording chamber. The chamber contained 1% agarose, in which the reference electrode was inserted. The isolated eye was positioned to face the light source. Under visual control via a standard microscope equipped with red illumination (Stemi 2000C; Zeiss, Oberkochen, Germany), a glass microelectrode with an opening of approximately 20 μm at the tip was placed against the center of the cornea. This electrode was filled with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4), the same in which the embryos were raised and held. A custom-made stimulator was invoked to provide light pulses of 100 ms duration, with a light intensity of 7000 lux using a ZEISS XBO 75W light source and a fast shutter (Uni-Blitz Model D122; Vincent Associates, Rochester, NY, USA), driven by a delay unit interfaced to the main ERG recording setup. Electronic signals were amplified 1,000 times by a pre-amplifier (P55 A.C. pre-amplifier; Astro-Med, Grass Technology) with a band pass between 0.1 and 100 Hz, digitized by DAQ Board NI PCI-6035E (National Instruments) via NI BNC-2090 accessories and displayed via a self-developed NI LabVIEW program.

AON delivery in a retinoblastoma cell line

The WERI-Rb1 (ATCC HTB-169) retinoblastoma cell line was obtained from ATCC. WERI-Rb1 cells were cultured in RPMI-1640 medium (Gibco; #21875034) supplemented with 10% fetal bovine serum (Bio-West; #S1810-500). Cells were maintained by addition of fresh medium or replacement of medium every 3 to 4 days. Cells were transfected with QR-421a (Table 1) using Lipofectamine 2000 transfection reagent (Invitrogen; #11668019). A ratio of 2:1 (volume: weight) between Lipofectamine 2000 and the AON was used. Both Lipofectamine 2000 and AON were prepared in Opti-MEM. Lipofectamine 2000 mixture was added to the AON mixture and incubated for 20 min at room temperature before adding the transfection complexes to the cells. Cells were incubated for 24 h at 37°C. For gymnastic delivery, QR-421a was directly added to the medium without any transfection reagent. Cells were incubated with QR-421a for 48 h at 37°C before harvesting for analysis. Two samples were treated per condition, AON, which is not complementary to the Ush2a sequence but with similar chemistry and length as that of QR-421a, was used as a control.

PBMC immune assay

Buffy coats, the fraction of an anti-coagulated blood sample that contains most of the white blood cells and platelets following centrifugation of the blood (500 mL blood in 70 mL citrate phosphate dextrose coagulant), from 5 healthy human (consensual) blood donors, were obtained from Sanquin Blood Supply in Leiden (the Netherlands). PBMCs were isolated from each buffy coat within 24 h after blood collection. PBMCs were stimulated for 24 h with QR-421a at a concentration of 0.1 μM, 1 μM, and 10 μM; positive control R-848 (1 μM); or PBS (vehicle control) at 37°C under a 5% CO2 atmosphere. For every donor, all conditions were tested in triplicate in 96-well round-bottom microtiter plates. The total number of viable PBMCs per well was 105. R-848 (Resiquimod; InvivoGen; thrL-R848), a potent

version (v.)1.47 software. First, the area of the connecting cilia was selected and manually isolated from the picture based on the centrin immunofluorescence signal. Subsequently, a mask was made based on the centrin staining using the centrin mask and layer containing the usherin immunofluorescence, the exact location of usherin immunofluorescence within the centrin mask. The resulting mask was dilated three times, and touching objects were separated using the watershed option. Subsequently, the maximum gray value of the identified regions was measured on the original image of usherin immunofluorescence ("analyse particles" option; size = 0–50, pixel circularity = 0.00–1.00).
Toll-like receptor (TLR)7/8 agonist, was selected as a positive control for its strong and robust immune-activating properties, inducing the production of pro-inflammatory cytokines. Also, R-848 acts upon the TLRs that are most likely to be involved in recognition of single-strand RNA, arguably making it the most relevant positive control for this purpose. After incubation, cell-culture supernatant was isolated following centrifugation (300 relative centrifugal force [RCF], 5 min, room temperature) for cytokine analysis. Cytokine levels in PBMC culture supernatants were measured using the MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel-Custom 12 Plex-Immunoassay Multiplex Assay (Millipore; #HCYTOMAG-60K). Analytes included were the following: interferon (IFN)-α2, IFN-γ, interleukin (IL)-1β, IL-10, IL-12 p70, IL-6, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, and tumor necrosis factor (TNF)-α. Assay plates were read on the Luminex MAGPIX platform (Luminex, San Francisco, CA, USA). Analysis of the Luminex data was performed in Bio-Plex Manager 6.1 software (Bio-Rad). Standard curves were fitted using 5 parameter logistic regression. Cytokine concentrations that were below the limit of detection (LOD), rendered “out of range <” by the analysis software, were imputed with a concentration value of $1/2^\text{LOD}$ for calculation purposes and statistical analysis. The LOD values, which were empirically determined by the manufacturer of the Luminex kit, were derived from the technical data sheet. Statistical analysis of the cytokine data was performed using GraphPad Prism 7 software. Geometric means of replicate cytokine concentration values were first log transformed.

### Differentiation and AON treatment of iPSC-derived PPCs

Following informed consent, a skin biopsy was obtained from a homozygous USH2A c.2299delG patient, and a primary fibroblast cell line was generated as previously described. Fibroblasts were reprogrammed using 4 lentiviruses expressing Oct3/4, Sox2, Klf4, and c-Myc. iPSC lines were generated on feeder cells (mouse embryonic fibroblasts) and subsequently maintained in Essential 8 medium (Life Technologies; #A1517001). After reaching confluence, iPSC clumps were digested with Accutase (Sigma-Aldrich; #A6964) and plated in a 12-well plate to form a monolayer. Upon reaching confluence, Essential-Flex E8 medium (Thermo Fisher Scientific; #A2858501) was changed into a differentiation medium consisting of DMEM/F12 ( Gibco; #11320-033), supplemented with non-essential amino acids (NEAAs; Gibco; #11140-050), B27 supplements (Thermo Fisher Scientific; #1287010), N2 supplements (Thermo Fisher Scientific; #17502048), 100 ng/μL insulin-like growth factor-1 (IGF-1; Sigma-Aldrich; #I3769), 10 ng/μL recombinant fibroblast growth factor basic (BFGF; Sigma-Aldrich; #F0291), 10 μg/μL heparin (Sigma-Aldrich; #H3149-10KU), and 200 μg/mL human recombinant COCO (Bio-Techne; #3047-CC). The medium was changed every day for 90 days, after which, the cells were treated with different concentrations of AONs for 1 month. At the end of the 4th month, the cells were collected and characterized. PPCs were treated continuously with 1, 2, 5, or 10 μM QR-421a or 10 μM control oligo, after 90 days of differentiation, for 28 days. Every 2 days, 50% of culture medium was refreshed with fresh culture medium containing AON. qRT-PCR was used to evaluate the differentiation status at the end of the experiment. Total RNA was isolated from PPCs as described for the human USH2A transcript analysis. 1 μg of RNA was reverse transcribed using Superscript VILO (Life Technologies; #117560650). Expression of NANOG, CRX, NRL, OPN1SW, OPN1LW, and RHO was investigated using 10× diluted cDNA using GoTaq DNA polymerase (Promega; #M3001) and a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). Data are normalized for the expression of the housekeeping gene GUSB. Primer sequences are provided in Table 2.

### Human USH2A transcript analysis

Total RNA was isolated from the cells using the RNeasy Plus Mini Kit (QIAGEN; #74136), according to the manufacturer’s protocol, and cDNA was synthesized. To visualize AON-induced alternative splicing in WERI-Rb1 cells and human iPSC-derived PPCs, a PCR was performed using primers on USH2A exons 11 and 15. PCR fragments were gel extracted, purified using the NucleoSpin Gel and PCR Clean-up Kit (Macherey-Nagel; #740609.50), and subjected to Sanger sequencing. For the quantification of USH2A transcripts, RT-ddPCR was performed using the One-Step RT-ddPCR Advanced Kit for probes (Bio-Rad; #1864022). The final 20-μL reaction mix contained the following: 5 μL Supermix, 2 μL RT, 1 μL 500 mM DTT, 1× TaqMan gene-expression assays, or 450 nM USH2A forward and reverse primer each and 250 nM USH2A probe. Total USH2A (Hs01071797_m1; Applied Biosystems) and USH2A exon 13 (Hs01071797_m1; Applied Biosystems) levels were quantified using 50 or 100 ng RNA, respectively, in a multiplex manner using commercial TaqMan gene-expression assays. USH2A Δ exon 13 levels were quantified in 50 or 100 ng RNA using an in-house-designed assay (Table 2). PCR reactions were dispersed into droplets using the QX200 droplet generator (Bio-Rad) according to the manufacturer’s instructions and transferred to a 96-well PCR plate. End-point PCR was performed in a T100 Thermocycler (Bio-Rad). The fluorescence of each droplet was quantified in the QX200 droplet reader (Bio-Rad). Each sample was analyzed in duplicate. Absolute quantification was performed in QuantaSoft software (Bio-Rad). Thresholds were manually set to distinguish between positive and negative droplets.

During the data analysis, average copy numbers of duplicate measurements per nanogram RNA input were calculated and normalized for total USH2A by dividing the target gene copy numbers by the total USH2A copy numbers. Percentage of USH2A Δ exon 13 transcripts was expressed relative to the amount of exon 13-containing transcript of untreated samples. In case untreated samples were not available, control-treated samples were used. This method was used as multiple USH2A isoforms are present in the cells, and the splicing modulation may not only lead to exon 13 skipping (e.g., combined exon 12 and 13 skipping, partial exon 13 skipping).

### Mouse IVT injection, necropsy, and collection of retinae

Animals were anesthetized using an intraperitoneal injection (10 μL/g body weight) of ketamine (10 mg/mL) and xylazine (0.5 mg/mL). Both eyes were then dilated with a topical mydriatic eye-drop mixture (1 drop each of tropicamide [1%] and phenylephrine hydrochloride...
cornea during recovery. Mice were sacri-
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ced with eye ointment (Duodrops; Medpets) to protect the
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eyed at looking for signs of intraocular bleeding. Both eyes were
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ection needle was retracted, and vascular integrity was exam-
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ined by followed an anterior counter-punch in the cornea before extracting
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ye was rinsed with ethanol and PBS between animals. For each injection,
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ap needle (VWR; HAMI7803-04) attached. The injection equipment
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urred to a 96-well PCR plate (SOP-EQP-028). End-point PCR was
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roduced into droplets using the QX200 droplet generator
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 and reverse primers each and 250 nM probe. PCR reactions
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s with the Bioanalyzer (Agilent) using the Agilent DNA 7500
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. For the quantification of Ush2a transcripts, One-Step RT-ddPCR
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ation mix contained the following: 5
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ndn and 95 nM forward and reverse primers each and 250 nM probe.
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a (measured at exon 8-9) copies.

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**Visualization of mQR-421a localization in the mouse retina**

A fluorescent in situ hybridization (FISH) assay was used for the detection of mQR-421a in murine ocular tissues to investigate localization and distribution. Eyes were collected and fixed in Hartmann’s fixative overnight at room temperature. The next day, lenses were removed, and the remaining eyeball was immediately processed to parafilm. Sections were cut and mounted on glass slides. Slides were hybridized with a fully complementary (to mQR-421a) Cy5-labeled, partly locked nucleic acid (LNA)-modified-probe (Cy5 5’-GATT 3’). DAPI containing medium was used to coverslip the slides and stain the nuclei. Microscopic images were taken using a confocal laser-scanning microscope (LSM, LSM800; Zeiss) using ZEN Blue software (Zeiss).

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.ymthe.2021.04.024.

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AUTHOR CONTRIBUTIONS
Conceptualization, K.D., H.C.v.D., E.d.V., J.J.T., G.P., P.A., and E.v.W.; writing, K.D., H.C.v.D., R.S., Ed.V., and E.v.W.; investigation and analyses: zebradish experiments, R.S., M.D., J.Z., S.B., T.P., and E.d.V.; 3D modeling, H.V.; cell culture, PPC, and mouse experiments, S.A., R.P., H.L.C., I.A.S., L.B., I.S., and J.M.; supervision, K.D., S.C.F.N., H.K., W.B., J.J.T., L.V., C.d.B., G.P., P.A., and E.v.W.; funding acquisition, E.v.W.; review, editing, and approval of manuscript, all authors.

DECLARATION OF INTERESTS
International patent applications have been filed by Stichting Katholieke Universiteit Nijmegen (WO2016/005514) and ProQR Therapeutics (WO2018/055134) describing methods and means regarding oligonucleotide therapy for USH2A-associated retinitis pigmentosa. Stichting Katholieke Universiteit Nijmegen has licensed the exclusive rights of the patent to ProQR Therapeutics. As the inventor, E.v.W. is entitled to a share of any future royalties paid to Stichting Katholieke Universiteit Nijmegen (WO2016/005514) and ProQR Therapeutics (WO2018/055134). E.v.W. is the inventor of an USH2A pseudoexon: implications for diagnosis and therapy. Hum. Mutat. 35, 1179–1186.

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